Preparation and characterization of a protein extract from date palm fruit (*Phoenix dactylifera* L.): investigation of nutritional and functional properties

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Submitted for the degree of

Doctor of Philosophy (Food Science)

Heriot-Watt University

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Edinburgh

January 2020

ABSTRACT

The world's population will reach 9 billion by 2050, increasing the pressure to find alternative protein sources to animal protein. The high global demand for soy protein ingredients has resulted in tropical deforestation which is associated with adverse health impact, agronomic, environmental and climate damage and there is a need to find alternative plant protein sources to soy protein. The Kingdom of Saudi Arabia (KSA) is considered as the second largest producer of date palm fruit among the current date-producing countries, resulting also in significant quantities of dates going to waste. The aims of this study were to develop a process for extraction of protein from date fruit suitable to be implemented in the food industry, to characterise the protein's electrophoretic profile by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), to identify the proteins by Liquid-Chromatography Coupled Tandem Mass Spectrometry (LC-MS/MS), to study the physicochemical and functional properties of date fruit protein extract (DFPE) and the effect of thermal treatment, to determine the chemical composition, nutritional value, anti-nutritional factors and anti-oxidant properties and to develop an infant cereal with reduced allergenicity.

Proximate and mineral analysis, anti-oxidant, amino acid composition and digestibility analysis of DFPE, reported in **Chapter 2** showed that the protein extract contains all essential amino acids, is a high source of iron and has excellent anti-oxidant properties matching that of ascorbic acid. The extract had a lower protein digestibility-corrected amino acid score (PDCAAS) value than soy protein isolate (SPI) and contained anti-nutritional factors e.g. oxalate, tannin and phytate, yet at low quantities that is assumed not to be of anti-nutritional concern.

Physicochemical and functional properties of the extract and the respective effect of thermal treatment are reported in **Chapter 3.** The concentration of free and total sulphydryl groups (FSH and TSH) were significantly less that for SPI, confirming the results of low cysteine in the amino acid analysis of DFPE. The effect of thermal treatment on the profile of sulphydryl (SH) groups indicates that DFPE is less thermally stable than SPI, whilst considering the fact that DFPE had been subjected to heat treatment during the extraction process. This physicochemical profile was mirrored by the corresponding decrease in functionality including decreases after longer heating times in solubility, foam capacity emulsion stability index and increased water separation in emulsions.

Chapter 4 describes the development of a protein extract containing 25.8% protein per dry weight using a new extraction process, which is a 13-fold enrichment compared to the 2.8% in the initial sample. The extraction process resulted in 4.2% DFPE, 57.2% date syrup and 38.6% waste. DFPE contains 50% protein whereas the remainder was in the date syrup (26.7%) and in the waste (24.3%) which could also be lost due to protease activity. The electrophoretic profile was established. LC-MS/MS results indicated the two most abundant proteins to be sorbitol dehydrogenase-like with MW (kDa) 39, an energy-related protein and catalase isozyme 2 with MW (kDa) 57, a disease/defense-related protein.

Chapter 5 describes the development of an infant cereal based on date fruit protein as a potential competitor to a commercial infant cereal (CERELAC) sold in the KSA. The date cereal product lacked certain amino acids and calcium and contained low levels of oxalate, phytate and tannin. A date-cereal porridge prepared with camel milk had a proximate and mineral composition matching that of a porridge made with CERELAC prepared with cow's milk apart from lacking fat, calcium and selenium, resulting in a product with reduced allergenicity to gluten and cow's milk. DFPE could add to the

rising need to find replacement for soy protein because the expansion of soy bean production and consumption is associated with environmental threats, such as deforestation.

DEDICATION

This humble work is dedicated to my dear late father *Mohammed*, my mother *Zahrah*, my beloved Husband *Yousef* and my kids (*Jamal, Amjad* and *Jenan*).

And above all to the almighty God

ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to *God* for supporting and helping me to complete my study and being with me in my whole life.

I must thank my government, represented by the *Saudi Cultural Bureau* in London and *Umm Al-Qura University in Makkah* for providing me with the scholarship and fully funding my study and living expenses as well.

Special thanks to my prime supervisor, *Dr. Lydia Campbell,* from the School of Engineering and Physical Sciences (EPS), Heriot-Watt University, Edinburgh who guided and helped me to complete this project through her continuous and powerful supervision: thanks so much for allowing me to grow as a researcher in the field of food science. I would also like to express my thanks to my second supervisor *Dr. Stephen Euston,* for his advice and encouragement in various ways throughout this study.

I am deeply indebted to my late father *Mohammed* and my dear mother *Zahrah* for their tears, support, encouragement and their immeasurable prayers for me. To my beloved husband *Yousef,* I could never thank him enough for his cooperation, support, patience and for providing me with unlimited positive energy and emotional support during my life, especially during this long journey. Words are powerless to express my gratitude to my wonderful sons *Jamal, Amjad* and my princess *Jenan,* who suffered from missing me while I was busy with this study. I am very grateful to my brothers *Taher, Mola, Abdul Shakur, Saleh and Hashem* who always stood behind me to support me whenever I needed it. I must thank my sisters, especially *Awatif,* my late sister *Fatema* and all beloved family and friends: thank you for your prayers and support to achieve my academic goals.

I wish also to thank my lab co-workers and especially, *Claire Munialo* (a Research Associate) and *Hector Mora* for their help and guidance pertaining to technical and scientific aspects of my project. Finally, I would like to express my gratitude and appreciation to *Mrs.Vicky Goodfellow* for her help and to *Professor Cait MacPhee* in the

School of Chemistry at the University of Edinburgh for carrying out LC-MS/MS analysis for the DFPE sample.

DECLARATION STATEMENT

HERIOT WATT UNIVERSITY

Research Thesis Submission

Name:	**Huda Mohammed Al-Barnawi**		
School:	**School of Engineering and Physical Sciences (EPS)**		
Version: *(i.e. First, Resubmission, Final)*	**Final**	Degree Sought:	**Doctor of philosophy (Food Science)**

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2. Where appropriate, I have made acknowledgement of the work of others
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Date Submitted:	

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DECLARATION

I *Huda Mohammed Al-Barnawi*, hereby declare that I am the author of this thesis. All the work described in this thesis is my own except where stated in the text. Results presented in this work have not been used in any previous application for a higher degree. All sources of information have been consulted by myself and are acknowledged by means of references.

Huda Mohammed Al-Barnawi

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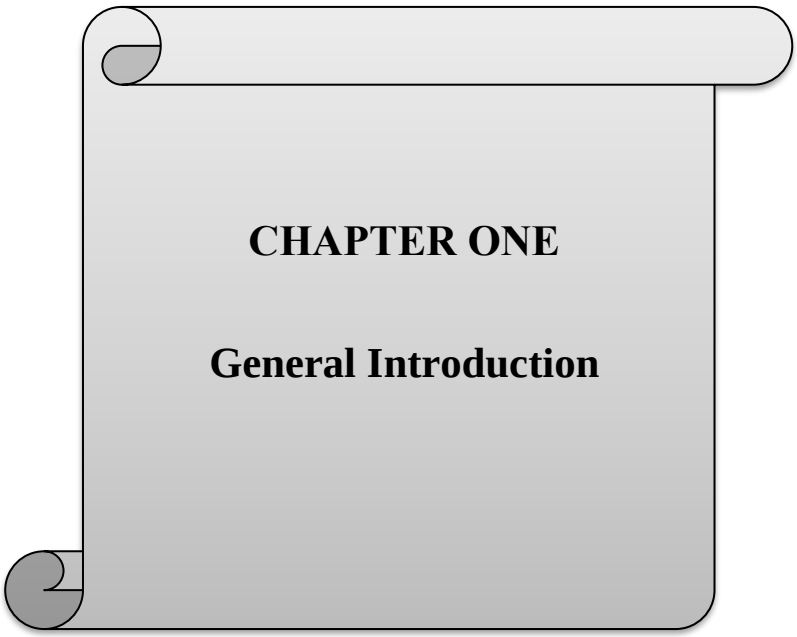
ABBREVIATIONS

µMol	Micromol
µl	Microliter
µg	Microgram
ABTS	2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)
AI	Adequate Intakes
AOAC	Association of Official Analytical Chemists
aw	Water activity
AAS	Amino acids score
ANS	8-anilino-1-naphthalenesulfonic acid
CERELAC	Brand of instant cereal made by Nestlé
DF	Dietary fibre
DFPE	Date fruit protein extract
dH_2O	Distilled water
DPPH	Radical Scavenging Effect Assay
DPP	Days post-pollination
DW	Dry weight
DTNB	5,5′-dithio-*bis*-2- nitrobenzoic acid
EAI	Emulsifying Activity Index
ESI	Emulsion Stability Index
EAA	Essential amino acids
FC	Foam capacity
FS	Foam stability
FSH	Free sulfhydryl groups
FAO	Food and Agriculture Organization
FRAP	Ferric Reducing Antioxidant Power
FTC	Ferric thiocyanate
HPLC	High performance liquid chromatography
IVPD	*In vitro* protein digestibility
KSA	The Kingdom of Saudi Arabia
kDa	Kilo Dalton
LAA	The most limiting amino acid
LC-MS/MS	Liquid-chromatography coupled tandem mass spectrometry
mMol	Millimolar
ml	Millilitre
M	Mole
mM	Millimolar
nm	Nanometre (unit of wavelength)
$NADP^+$	Nicotineamide adenine dinucleotide phosphate (oxidized form)
NCBI	National Center for Biotechnology Information
NHS	National Health Service
ORAC	Oxygen Radical Absorbance Capacity
OAC	Oil Absorption Capacity
O/W	Oil-in-water emulsions
pH	Decimal logarithm of the reciprocal hydrogen ion activity
pI	Isoelectric point
PBS	Phosphate Buffered Saline
PEM	Protein-energy malnutrition
PEPC	Phosphoenolpyruvate carboxylase
PMSF	Phenylmethylsulfonyl fluoride

PDCAAS	Protein digestibility corrected amino acid score
RNI	Reference Nutrient Intake
RT	Room temperature
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SCAR	Protein identified in DFPE
SPI	Soy Protein Isolate
TCA	Trichloro-acetic Acid
TNB	2-nitro-5-thiobenzoic acid
TPTZ	2,4,6-Tri (2-pyridyl)-s-thiazine,
TSH	Total sulfhydryl groups
USDA	United States Department of Agriculture
UV	Ultraviolet
UF	Ultrafiltration
v/v	Volume to volume ratio
v/w	Volume to weight ratio
V	Volt
WAC	Water Absorption Capacity
WHO	World Health Organization
w/w	Weight to weight ratio
w/v	Weight to volume ratio
W/O	Water-in-oil emulsions
WP	Water Phase



CHAPTER ONE

General Introduction

1.1 Introduction

Human beings have cultivated date palm fruit (*Phoenix dactylifera* L.*)* for food for more than 6000 years. It has been the principal produce grown in arid and semi-arid areas of the world, with the result that it has played a prominent function in the economic and political life of the people of these areas for the last 7000 years (Ahmed et al., 1995b; Besbes et al., 2004; Ashraf and Esfahani, 2011).

The key aim of this research is to extract protein from date palm fruit from The Kingdom of Saudi Arabia (KSA) in order to produce an ingredient for food applications. The main reasons for selecting this topic is as follows:

- The world's population will reach 9 billion by 2050, increasing the pressure to find alternative protein sources to animal protein which is associated with adverse health impact, agronomic, environmental and climate damage (European Commission, 2015; Henchion et al., 2017).
- There is a need to find alternative plant protein sources to soy protein (European Commission, 2018); the high global demand for soy protein ingredients has resulted in tropical deforestation (Union of Concerned Scientists, 2019).
- KSA is considered as the second largest producer of date palm fruit among the current date producing countries in 2011 the number of date palm trees was estimated to be over four million and around 1000,000 tons of dates are being produced annually (Basuny and Al-Marzooq, 2011). Most date processing factories in the KSA focus on processes, such as pressing and packaging dates, removing the seeds and filling them with nuts, rather than manufacturing new added value products. In the past few years, several factories have started producing date pastes. However, these factories produce only date honey (dibis), vinegar, jams, date nectar and animal foods from the waste of date fruit and seeds (Chandrasekaran and Bahkali, 2013). In addition, these products are being

produced by a limited number of factories and in limited quantities. Moreover, there is an absence of manufacturing research centers which require sustained funding to conduct research to improve manufacturing and packaging and produce new products from dates. There is also a lack of knowledge and know how regarding the engineering, chemical and microbiological aspects, as well as sensory evaluation and studies of the shelf life and promotion of new products. Due to the limited research relating to date fruit in the KSA, in comparison with the scale of date production and the associated non-utilized waste, there is a need for further research to extract protein as an added value ingredient. Moreover, relatively few research publications are available on the characteristics and functionality of protein of date palm fruits.

1.2 Objectives of the research

1. To determine the chemical composition, nutritional value, anti-nutritional factors and anti-oxidant properties of the protein extract.
2. To study the physicochemical and functional properties of date fruit protein extract (DFPE) and the effect of thermal treatment, using soy protein isolate SPI as control.
3. To extract proteins from one variety (Shalaby) of date fruit at the *Tamr* stage of maturity, comparing three different extraction methods.
4. To adjust and optimize an extraction procedure to produce an ingredient that is safe for human consumption.
5. To identify the proteins by Liquid-chromatography Coupled Mass Spectrometry (LC-MS/MS).
6. To characterise the proteins electrophoretic profile by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of date fruit protein extract (DFPE) using soy protein isolate as control.

7. To produce an infant cereal suitable for children to prevent development of allergies against gluten, lactose and bovine milk using commercial infant cereal as a control.

To achieve these objectives, the approach to the study have been divided into four sections.

1) Chapter 2 will cover objective 1
2) Chapter 3 will cover objective 2
3) Chapter 4 will cover objectives 3 to 6
4) Chapter 5 will cover objective 7

1.3 Novelty of the approach

1. Extraction of protein from date palm fruit at higher concentration than 2%, and determination of the chemical composition, nutritional, anti-oxidant and anti-nutritional properties.
2. Determination of physicochemical and functional properties and the effect of thermal treatment.
3. Development of an infant cereal suitable for children to prevent development of allergies against gluten, lactose and bovine milk.
4. Determination of chemical composition, nutritional value and water activity of the infant cereal product.
5. To calculate the nutritional value as a proportion of Reference Nutrient Intake (RNI) for children under 5 years of age.

1.4 Layout of the thesis

This first chapter of the thesis is a general introduction, while the second chapter describes the chemical composition and nutritional analysis of DFPE. The investigation of the physicochemical and functional properties of DFPE and the effect of heat treatment on those properties is reported in the third chapter. The fourth chapter presents all the information obtained from the work involving the isolation and characterisation of date palm fruit protein extract (DFPE). The fifth chapter deals with its application in development of an infant cereal product. General conclusions and recommendations for future study are presented in the chapter six.

1.5 Background literature study

1.5.1 Date Palm

1.5.1.1 General botanical information

The botanical name of the date palm is *(Phoenix dactylifera* L.*)*. The meaning of "phoenix" is date palm, and it is said to be derived from a Phoenician name, while the meaning of “dactylifera” is a finger, illustrating the fruit's form and is derived from the Greek word "daktulos" (Zaid and Wet, 2002). There is another explanation of this botanical name for date palm which is that it is derived from the legendary Egyptian bird, the "*Phoenix*", while "*dactylifera*" is derived from the Hebrew word "dachel" (Zaid and Wet, 2002). The date palm (*Phoenix dactylifera* L.) belongs to the family Arecaceae and the plant consists of five parts: the roots, trunk, leaves, flowers and fruits, as shown in **Figure 1.1**.

Roots

The date palm has a fibrous root system, originating from a bulb at the trunk base. The primary root has an average length of 4 m and up to 10 m in light soil (Manickavasagan et al., 2012). The primary root gives rise to secondary roots that further branch to create a tertiary root system (Zaid and Wet, 2002).

Trunk

The trunk of the date palm consists of a single columnar cylinder. Its height is around 30 m and it is brown in colour and striped. It is covered by the leaves’ bases, which are enclosed in fibre, which protects the trunk from herbivorous insects and animals and provides insulation to decrease water lost (Manickavasagan et al., 2012).

Leaves

The date palm tree produces around 10 to 26 leaves annually and has approximately 100 to 125 leaves in the mature stage (Zaid and de Wet 2002). The fully mature leaf of the date palm has an average length ranging between 3 to 6 m and 0.5 m wide each leaf

consists of 150 leaflets about 30 cm long and 2 cm wide. The leaf consists of three regions: i) the petiole and the spinal region, which transitions into ii) the blade region, which is attached to a geometrically shaped iii) midrib. The leaf is narrow at the midrib and towards each leaf end (Manickavasagan et al., 2012). Leaves can live between 3 to 7 years and remain attached to the tree until manually pruned or following their senescence.

Flowers

Date palm flowers grow from auxiliary buds between the terminal rosette and form in branched clusters. *Phoenix dactylifera* L. is a dioecious species: male and female flowers grow on separate plants and the female palms will bear fruit upon artificial or natural fertilisation. The flowers of the male and female are white, very small and organised in strands attached to a central stem referred to as the rachis, forming a spike or spadix (Zaid and de Wet, 2002).

Fruits

The date palm fruit is a single, oblong berry characterised by a terminal stigma, a fleshy natural covering and a membranous pit between the seed and the fruit (Zaid and de Wet 2002). The fruit consists of four parts: the seed, epicarp, mescarp and endocarp (**Figure 1.2)**. There are many types of date fruits, as illustrated in **Figure 1.3**, each type differing in size, shape, colour, and chemical composition, depending on varietal differences, soil, climate and growing conditions (Al-Yahyai and Kharusi, 2012).

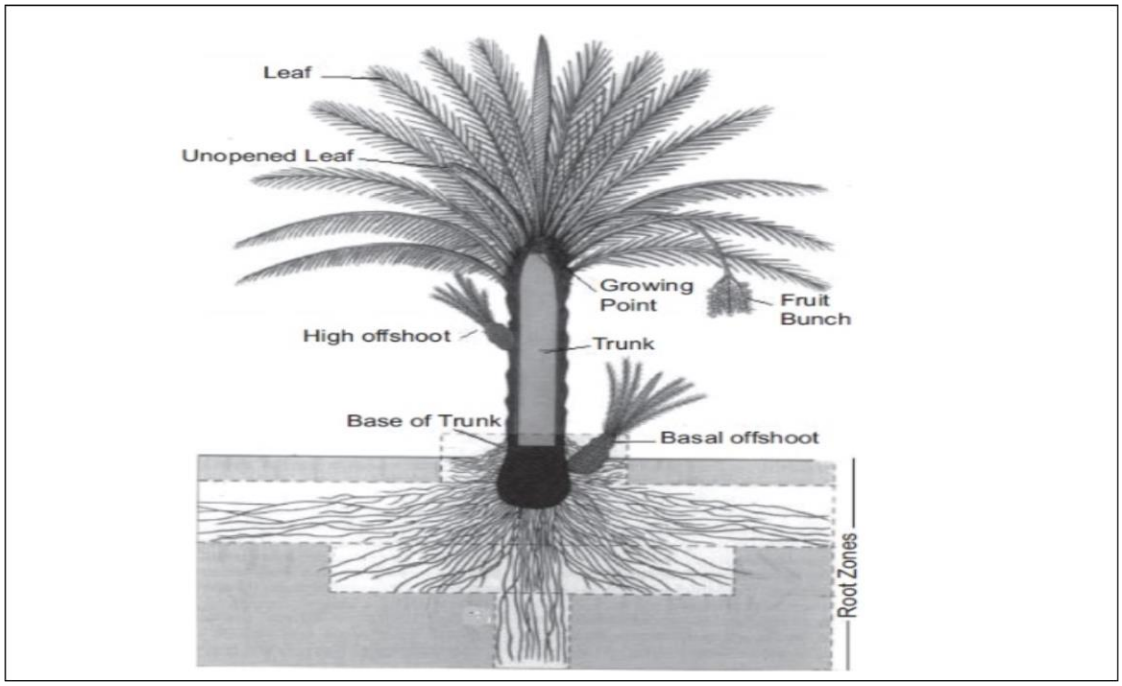


Figure 1.1: Date palm tree (Siddiq et al., 2014).

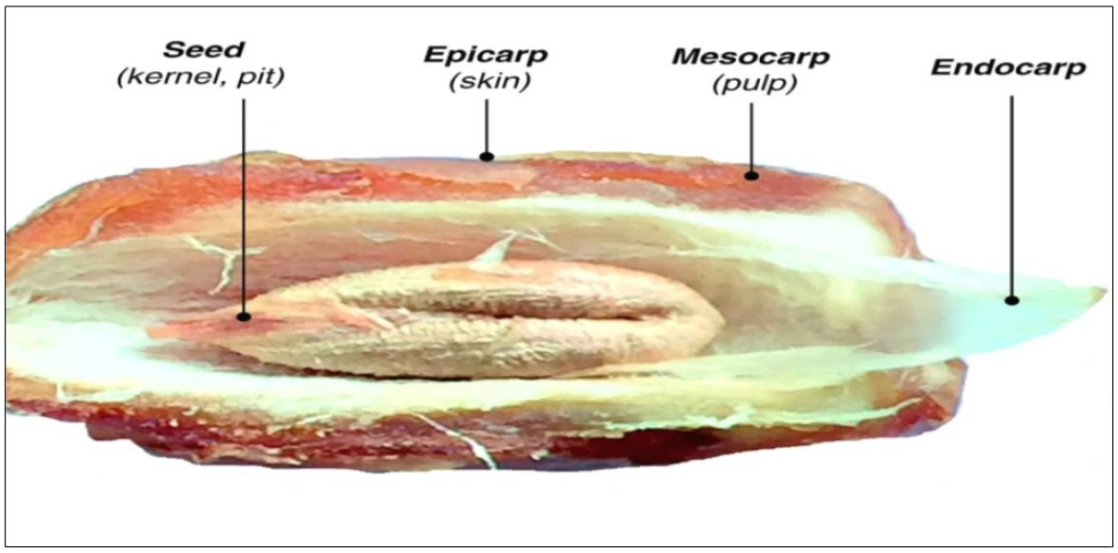


Figure 1.2: The dissection of the date palm fruit at the *Tamr* stage (Ghnimi et al., 2017).

Figure 1.3: Various types of date palm fruit (Siddiq et al., 2014).

1.5.1.2 Different stages of date fruit ripeness

The date palm fruit (*Phoenix dactylifera* L.*)* is amongst the important agricultural commodities in the Middle East, Asia, and North Africa. The fruit of date palms has served as a supply of wealth for several countries for many years as a stable nutrient food. Dates are sold around the world, traded as goods or processed into many different products (Khan et al., 2008). The maturity of a date fruit is usually classified into five stages, named by Arabic words which are internationally employed by numerous authors, e.g. Ahmed et al., (1995b); Al-Shahib and Marshall (2003); Kader and Hosain (2009); Baliga et al., (2011), as well as Israeli and American date growers. These stages are shown in **Figure 1.4** and are known as *Hababouk, Kimri, Khalal, Rutab* and *Tamr* (Ghnimi et al., 2017); there are no equivalent English words.

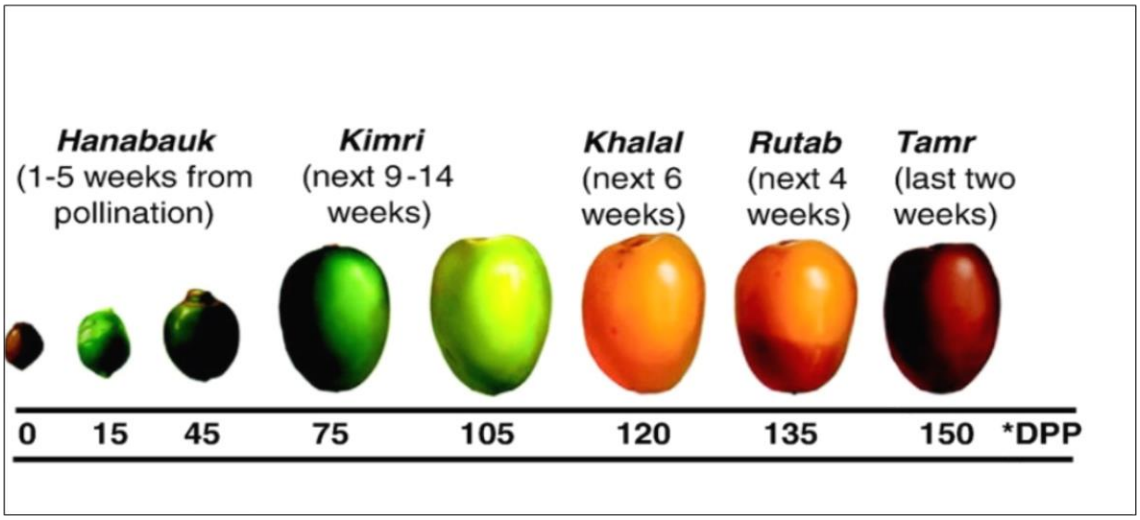


Figure 1.4: The stages of date fruit ripening according to days post-pollination (DPP) (Ghnimi et al., 2017).

The fruit becomes edible in the last three stages, due to its improved tenderness, succulence and increased sweetness and its decrease in bitterness. The *Hababouk* stage is the first stage of ripening of the fruit: during this stage it is green in colour, and growing slowly, taking between 4-5 weeks to finish the process of growth. The second stage of ripening is the *Kimri* stage, also called the green stage and in this stage the colour of the date changes from green to light yellow; it takes between 9-14 weeks to complete the growth process and it is not suitable for eating. The moisture content in this stage is 85%. The third stage is the *Khalal* stage, also called the coloured stage. In this stage the date changes from a green colour to yellow or red, which takes around 3-5 weeks to complete. At this stage dates can be consumed as fresh fruit, or they can be used for butter, jam or date-in-syrup. The moisture content is this stage ranges between 45 to 65%. The fourth stage is the *Rutab* stage, also called soft ripe stage: in this stage the colour of the date changes to light brown and it is soft in texture. It takes between 2-4 weeks to complete the process of ripening, when it is ready for consumption. The moisture content in this stage ranges between 30 to 45 %. The fifth stage is the *Tamr* stage, the fully ripe stage or last stage in the ripening: in this stage the colour of the fruit turns to dark brown or black. It is soft in texture and has lost most of the date water and it takes from 2-4 weeks to

complete the process of the ripening. It is now good for consumption and storage. The moisture content is <30% in this stage.

The texture of the date depends on the amount of pectin methylation: the lower the amount of methylation, the softer the date. The *Tamr* date is the softest (39% methylation) and the *Kimri* date is the toughest (72% methylation) (Rohani, 1988; Myhara et al., 2000). Date fruits can be processed into diverse products, such as date powder, syrup or paste, which can be used in manufacturing cake or biscuits (Ghnimi et al., 2017). The largest consumption of dates is at the *Tamr* stage, due to their good storability when dried making them available all year around. Some date varieties, such as *Khasab* and *Hilali* from Pakistan, do not produce the *Tamr* stage and those are consumed at the *Khalal* and *Rutab* stages (Ahmed et al., 1995b).

1.5.1.3 Storage of date palm fruit

The harvest season is during 2-3 months in the season of summer. As a result, many date factories may receive quantities of fresh dates at the *Tamr* stage during summertime, which exceeds the market demand. Therefore, there is a need to store the date fruit and re-sale it at later of the year (Ismail et al., 2008). Only date fruit at the *Tamr* stage can be stored for future processing or consumption (Al-Yahyai and Al-Kharusi, 2012).

Date fruit can be stored under refrigeration or freezing conditions to reduce enzyme activity (Biglari, 2009). The temperature of 0°C is the optimum storage temperature that allows a storage period of 6-12 months (Ashraf and Esfahani, 2011). The storage time of date depends on the variety; the soft varieties have less storage time than the semi-dry varieties. Date can be dried by using drying tunnels or solar drying (Ashraf and Esfahani, 2011). According to Biglari (2009) storing dates at -18°C has a negative effect as it accelerates date ripening and tissue injury (Sahari et al., 2007).

1.5.1.4 Worldwide consumption and production

World date production was about 6.9 million metric tons in 2011 and the top ten date producing countries were Egypt, KSA, Iran, Iraq, UAE, Pakistan, Algeria, Oman, Tunisia, and the USA, respectively (**Table 1.1**). Other significant producers include China, Morocco, Yemen, Israel, Kuwait, USA, Turkey, and Mauritania. Smaller quantities of dates are produced in India, Chad, Somalia and Mexico (Fao.org, 2015). Dates are marketed around the world as a high-value fruit crop. The relatively high production of dates in Egypt is attributable to the significantly higher tree density per hectare when compared to KSA, Iran, UAE, Algeria, and Iraq (Siddiq et al., 2014).

Asia is both the largest importer and consumer of dates; specifically, India and China are key importers of dates. The first and second largest importers of dates in the EU are France and Germany, respectively. The USA is the main importer of dates, followed by Canada (Hui, 2006). KSA is considered to have been the origin of the date palm, which is believed to have existed 10,000 years ago. Currently, the KSA is the second largest producer of dates, producing more than 300 types of date, each with its own texture and taste.

Table 1.1: Cultivation, production, export and export value of dates, according to the FAO Statistical Database (2007), cited in Al-Farsi and Lee (2008a).

Countries	Area 1000 Hectares	Production 1000 tons	Export 1000 tons	Commercial value US $/ton	Export value 1000 US $
Algeria	135	470	8	1,820	14,563
Egypt	35	1,166	3	457	1,370
Iran	185	880	95	383	36,430
Iraq	102	875	24	183	4,392
Oman	34	238	5	436	2,180
Pakistan	81	622	65	346	22,473
Saudi Arabia	145	901	44	548	24,090
Tunisia	45	122	40	2,110	84,382
UAE	186	760	60	219	13,127
USA	2	15	4	3,339	13,357
World	1,129	6,908	377	786	296,248

1.5.1.5 Uses of the date palm fruit

Many edible products can be made from dates, including date powder, date syrup, animal feed, alcohol, various sorts of sweets, bread, marmalade, chocolate and date paste (Ashraf and Esfahani, 2011). In some factories date syrup is added to yogurt to increased nutritional value and taste. It also used as a sweetening agent to replace glucose syrup, malt syrup, high fructose syrup, molasses, invert sugar and all other kinds of crystalline sugars (Al-Hooti et al., 2002). Date fruit can be eaten out-of-hand or pitted and stuffed or cut-up and used in various ways, such as in puddings, breakfast cereals, ice cream, cakes, bread or biscuits. In some Arab countries many types of beverages are made from the date fruit pulp, such as alcohol, known as 'arak', vinegar, and date juice, which is known as 'dibbis' or syrup "honey date" (Al-Turki, 2008). The ancient Egyptians used date fruit or its juice in many medicinal treatments. They also made

around seventeen kinds of beer and twenty-four forms of wine, a few which were used as ingredients of medicines (Al-Turki, 2008).

1.5.1.6 Uses of other parts of the date palm

Economically, other parts of the date palm make it a significant agricultural product. For example, the tree's leaves have been used for making handicrafts, such as straw hats and fans, and its trunk and branches can be used for covering the roofs of rural houses, or for making boats. It is also used in paper and wood industries (Fao.org, 2016). In the past, date seeds were mainly used to feed animals, such as cattle, camels, sheep, fish and poultry, but now the powder of date seed is used as a coffee and it was recently introduced to the market as the pure powder or in a mixture with coffee powder (Rahman et al., 2007; Al-Farsi et al., 2007). In some Arabian markets in the KSA and UAE, roasted date seed powder is used as coffee or substitute coffee; the date seed coffee has a lower content of total phenolic compounds than Arabic coffee (Ghnimi et al., 2017). In the Arab world the date seed, which would be a waste material, is used to make the caffeine-free drink. Date palm fruit and seed waste can also be used as an environmentally friendly composting method by treating a combination of 70% date fruit and seed waste with 30% of crab shell and shrimp waste to create fertiliser for plants (Khiyami et al., 2008).

Date palms are used in the manufacture of several products that are beneficial for human health. In addressing issues connected with obesity and being overweight, dates can be used to create tasty and fat-free snacks and will be possibly present in some of the most effective foods in the future (Al-Shahib and Marshall, 2003; Miller et al., 2002). The date palm has also long been planted for non-food uses, such as building materials, shade, fibre and fuel in the semi-arid and desert areas of the Middle East, India, Northern African, Pakistan, the USA, and the Canary Islands (Barreveld, 1993).

1.5.1.7 The issues of the waste of dates

KSA generates more than 200,000 tons of date palm biomass every year (Zafar, 2018) and more than 236,807 tons of dates are discarded each year in Algeria, resulting in environmental issues (Ahmed et al., 2016). Thus, there is an urgent need to find appropriate applications for this waste. The wastes of date fruit (as shown in **Figure 1.5**) particularly the seeds of dates and date press cakes (a by-product of date juice production) have a considerable scope for creating added value using bioprocess technologies (Chandrasekaran and Bahkali, 2013).

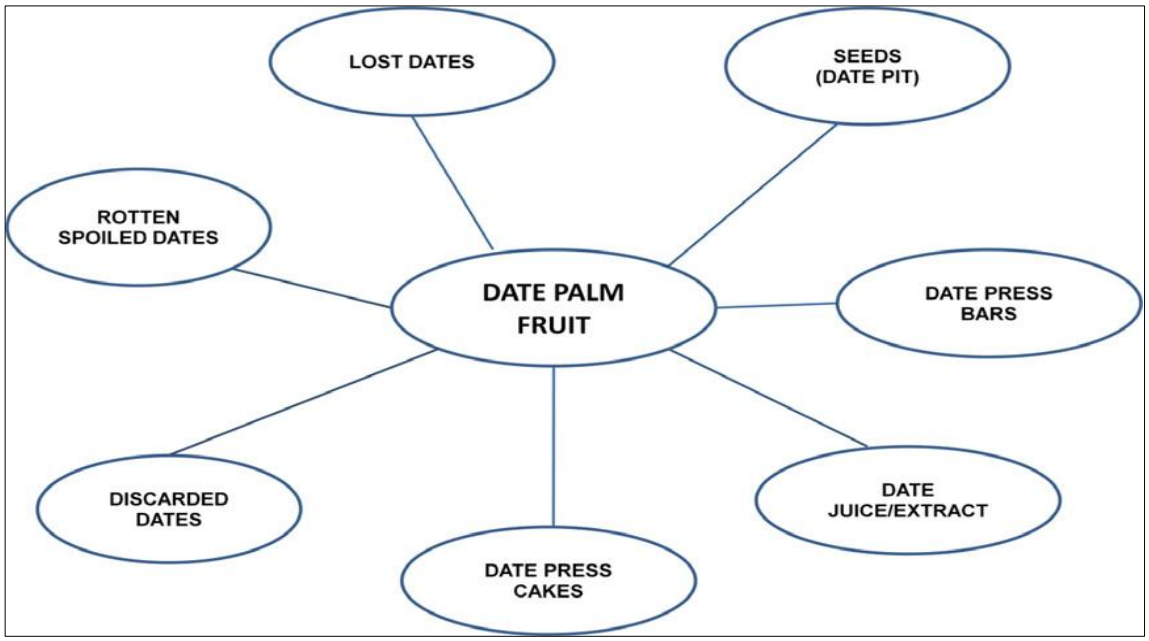


Figure 1.5: Date palm fruit by-products and wastes (Chandrasekaran and Bahkali, 2013).

1.5.1.8 Nutritional value of dates

Dates are considered a direct supply of all phenolic compounds and natural anti-oxidants, such as anthocyanins (Al-Farsi et al., 2007). Anti-oxidants that have been suggested to play a significant role in the prevention of cardiovascular disease, cancer, inflammation, and neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (Joseph et al., 1999; Clarke, 1999; Wargovich, 2000) and may also directly provide anti-cancer activity (Sun J-Cha and Wa, 2002).

Al-Mamary et al., (2010) reported that, in ancient medication, date fruit was suggested to be consumed by pregnant ladies before and during delivery to provide energy and also for treatment of liver diseases.

Consumption of dates may be of advantage in lipid management and glycaemia in diabetic patients (Miller et al., 2002). The high level of dietary fibre has been reported to have a preventative impact against several diseases, including obesity, hypertension, coronary cardiovascular disease, hyperlipidaemia, diabetes, prostate cancers, colorectal cancer and intestinal disorders (Tariq et al., 2000).

1.5.2 The importance of proteins in our diet

Proteins are an essential component of the human diet, as they are necessary to support muscle tissue and the normal growth and maintenance of the body. Twenty amino acids are generally found in proteins. Nine of these are primary need for human adults and eight of these need to be obtained from food because the body cannot create them. Essential amino acids can be defined as "Ones which cannot be synthesized by the animal organism out of materials ordinarily available to the cells at a speed commensurate with the demands for normal growth"(Reeds, 2000). These nine amino acids are known as essential amino acids and so must be provided by the diet in order to grow and preserve health (**Table 1.2)**. Conditionally essential amino acids are usually not essential, except in conditions of illness and stress. The synthesis of arginine, cysteine, glycine, glutamine, proline and tyrosine are limited under special pathophysiological conditions, such as in individuals with catabolic diseases and are therefore conditionally essential. Non-essential amino acids are those that can be created by the human body and can be defined as "ones that can be synthesized from a non–amino acid source of nitrogen, such as ammonium ions and an appropriate carbon source" (Reeds, 2000).

The nutritional value of a protein is determined by its amino acid composition. Based on the content of amino acids, there are two types of proteins: the first type is a complete

protein, and the second type is an incomplete protein. High quality protein comes from animal sources and contains all the essential amino acids that are required for the synthesis of protein and other important compounds containing nitrogen, such as hormones, peptides, creatine and some neurotransmitters (National Research Council, 1989). Low quality protein comes from plant sources and lacks one or more essential amino acids (Hoffman and Falvo, 2004).

Table 1.2: Different classes of amino acids

Essential amino acids	Conditionally essential amino acids	Non-essential amino acids
Histidine	Arginine	Arginine
Isoleucine	Cysteine	Cysteine
Leucine	Glycine	Glycine
Lysine	Glutamine	Glutamine
Methionine	Proline	Proline
Phenylalanine	Tyrosine	Tyrosine
Threonine		Alanine
Tryptophan		Asparagine
Valine		Aspartate
		Serine

1.5.3 Background of malnutrition in children under 5 years old

Balanced nutrition is an important element for human development (Smith and Scholey, 2014). Without it, people will suffer from some diseases, decreased productivity, immunity, and development (The World Health Organization, 2016). The World Health Organization (WHO) defines malnutrition as ‘‘the cellular imbalance between the supply of nutrients and energy and the body’s demand for them to ensure growth, maintenance, and specific functions’’ There are two types of malnutrition: chronic malnutrition and acute malnutrition. Chronic malnutrition happens when children do not meet the standard height and weight requirement for their ages (Humanium, 2016). There are several chronic diseases associated with malnutrition, such as chronic renal failure, neuromuscular diseases and congenital heart disease (Martore et al., 1992). The prime symptom of malnutrition is emaciation which can be defined as a body weight that is 25%

less than the expected normal (Goette, 2005). It happens when the body lacks a large amount of essential fat and muscle tissue (Stice et al., 2004). Inadequate intake of calories can also lead to weakness of the immune system, changes in mood and feeling of low energy (Goette, 2005). Protein-energy malnutrition (PEM) can lead to the body losing more than 60% of its normal weight and is associated with an abdomen that is distended while the upper ribs are prominent (Goette, 2005). Based on the World Health Organization Global Database on Child Growth and Malnutrition (De Onis and Blössner, 2003) 87% of the total population of under-5-year-olds in developing countries suffer from PEM especially those living in southern Asia 5% in Africa, and 5% in Latin America. One study has shown that malnutrition was directly responsible for up to one-third of all child deaths in the world and nearly two billion people all around the world that suffer from malnutrition (Scientific Advisory Commission on Nutrition, 2015; The WHO, 2016). In Africa, particularly in Somalia, hunger and malnutrition lead to economic losses as well as human devastation (Gettleman, 2012). Malnutrition is also prevalent in displaced people and refugees. Based on reports of the WHO, 21.5 million displaced people as well as refugees in different countries of the world are suffering from the problem of malnutrition (World Health Organization, 2009); around 2,000 children of Syrian refugees in Lebanon are suffering from severe acute malnutrition, and they need urgent treatment to survive. There are many Syrian refugees at the risk of anemia, a condition where the oxygen carrying capacity of red blood cells, or the number of red blood cells, is inadequate; one of the major causes being lack of iron in the diet (Unicef, 2014). There is no published information on the prevalence of malnutrition in Saudi Arabia (El Mouzan et al., 2010).

1.5.4 Food allergies

Food allergies can be defined as “an adverse health effect arising from a specific immune response that it reproducible upon subsequent exposures to a given food” (Boyce et al., 2010). The prevalence of food allergies is increasing worldwide as documented by some studies in The United Kingdom (Turner et al., 2015), Australia (Mullins et al., 2015) and The United States (Rudderset al., 2014). However, Boyce (2012) reported that there are rare studies and information regarding the prevalence of food allergies in developing countries including The Kingdom Saudi Arabia. Food allergies can be classified into two types, which are plant food allergies and animal food allergies. Generally, milk, eggs, soy, wheat, peanut, tree nuts, fish, and shellfish are considered the most ingredients that cause food allergens in humans but the most common causes in an infant are cow’s milk and egg (Mehta et al., 2013).

The most critical period of growth and development in childhood is during their first 2 years of life during which most allergies develop, leading to poor growth and insufficient intake of nutrients, especially those who avoid having cow’s milk (Mehta et al., 2013). The major approach to manage of food allergies is the avoidance of certain types of food that leads to insufficient food intake and malnutrition (Meyer et al., 2013).

1.5.5 Protein resources

Animal-based protein are obtained from sources such as chicken, beef, fish and eggs. The price of meat is very high in developing countries; therefore, it is important to find other sources of protein instead of meat protein.

Plant proteins are derived from soybeans, corn, canola, sorghum, rice, millet, and wheat (Ustunol, 2015). Some plant proteins lack one or more amino acid in sufficient quantity and can be addressed by consuming a combination of different proteins that complement each other (McDougall, 2002). The main advantage of plant proteins is their lower prices compared to animal sources. Especially in developing countries, around 90% of the

population's protein intake is derived from plant sources (Salunkhe and Deshpande, 1991). Cereals, such as millet, wheat, sorghum, rice, and rye are an important source of protein worldwide; they contain a high level of starch and a medium level of protein, at about 8-14% and have low lipid content. Soy protein is one of the most widely consumed oilseed proteins in the world. The main reason for the consumption of soy protein is its low-cost relative to meat proteins and its high protein content (Ustunol, 2015).

Alternative proteins include sources, such as microalgae, bacteria and mycoprotein, as well as future alternatives, such as synthetic or laboratory-grown meat.

1.5.6 Reasons for finding a new source of proteins

There are many factors driving the increase in food production of in the world and the search for different kinds of food: these factors are the increasing world population together with demographic and social change. Moreover, the demand for animal protein will increase and it is expected to have a negative effect on the environment, leading to greenhouse gas emissions, requiring more land and water (Henchion et al., 2017).

By 2050 the world's population will increase from 2000 to 9.5 billion (Henchion et al., 2017) **Figure 1.6**. The projected world demand for animal-derived protein will double by 2050 (Westhoek et al., 2017), resulting in concerns for sustainability and food security.

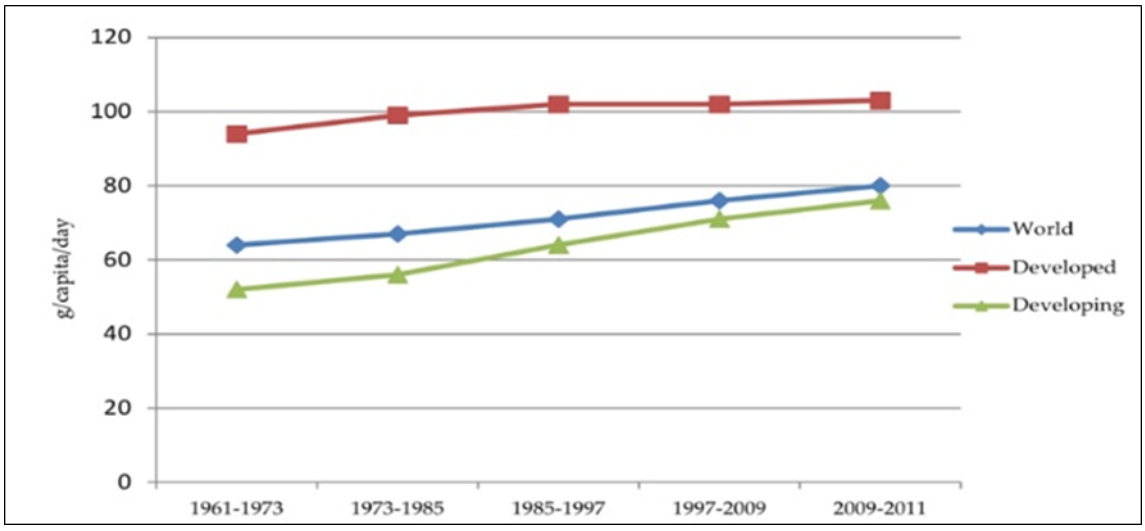


Figure 1.6: Evolution of animal protein consumption per capita (g/capita/day) (Henchion et al., 2017).

Analysis of this data by Henchion et al., (2017) found that overall meat consumption increased by almost 60% between 1990 and 2009. This trend is expected to continue driven by income growth in countries, such as Asia, Latin America and the Middle East. Protein from meat is an excellent source of essential amino acids and has high net protein utilisation and digestibility (Bax et al., 2013). In addition, meat is a key source of often highly bioavailable minerals (iron, zinc, and selenium) and vitamins (A, B9 and 12, D, and E), and has an ability, referred to as the "meat factor", to enhance iron availability from other sources (Hurrell and Egli, 2010).

From an environmental perspective, meat production, at a global level, contributes significantly to climate change and land use change (Hurrell and Egli, 2010). High levels of greenhouse gases (GHG) are produced during meat production, with ruminants contributing in a significant way. Land use, water, energy and chemical inputs (e.g., fertilizers) all reflect negatively on the environmental footprint from meat production.

Increased demand for protein globally is driven by socio-economic changes, such as rising incomes, increased urbanisation, aging populations, where the contribution of protein to healthy aging is increasingly recognised as part of a healthy diet (Delgado, 2003; Popkin et al., 2012).

The global demand for soy protein ingredients is increasing due to finding plant protein sources to replace animal proteins. To date soy protein is the major functional protein used owing to its excellent functional properties, such as emulsifying ability and neutral sensory properties, which other alternative sources fail to match. In the UK, soy imports have grown from 2.7 million tonnes in 2011 to 3.8 million tonnes in 2015, while in the KSA, soybean meal imports have increased from 9 million tons in 1976 to 1175 million tons in 2018 (Indexmundi.com, 2019). Argentina, Brazil and the USA account for more than 80% of global production of soybean (Bothends.org, 2014). The expansion of soy production and consumption has been associated with deforestation and another natural

habitat destruction (Nepstad et al., 2006). Excessive use of agrochemicals to produce soybean is a major environmental threat because of causing water contamination, which could pose widespread health risks to people living near soy farms (Bothends.org, 2014). These factors give ample evidence for the global need to find alternative plant protein ingredients.

1.5.7 Religious and cultural significance of the date fruit

The date palm fruit is recognised as an important fruit for Muslims around the world. Traditionally, date palm fruit is used to break the fast for Muslims throughout the month of Ramadan (Al-Shahib and Marshall, 2003; Al-Farsi and Lee, 2008a). The nutritional significance and health benefits of dates are mentioned in 20 verses of 17 Surah's (chapters) in the Holy Quran (Manickavasagan et al., 2012). Prophet Mohammed urged Muslims to eat date palm, maintain dates or chards and mentioned that dates can cure many diseases.

Similarly, the date fruit is praised in Judaism and the Christian religion and is associated with various religious ceremonies, like Pesah and Palm Sunday (Musselman, 2007). There are many references to the date palm in the Bible, praising its manifold virtues. In Judaism, dates are considered one of the seven holy foods/seeds.

CHAPTER TWO

Chemical composition and nutritional analysis of date palm fruit protein extract

2.1 Introduction

Dates have a high nutritional value and could play an efficient role in providing the nutritional needs of humans. Each kilogram of fresh dates contains around 1570 calories of energy, while dry dates contain over 3000 calories per kilogram (Rohani, 1988).

This chapter reports on the third area of the present research, which involves presenting the results of the chemical composition of DFPE, the determination of essential and non-essential amino acids, *in vitro* protein digestibility (IVPD), the amino acids score (AAS) and protein digestibility-corrected amino acid score (PDCAAS). These results are reported for the first time for proteins in date fruit.

Further nutritional analysis reported here includes vitamins, minerals, anti-oxidants and anti-nutritional factors of DFPE.

2.1.1 Chemical composition and nutritional value of date palm fruit

The compositional characteristics of date fruit are important, as the chemical components are associated with nutritional value and health advantages. The composition of dates may be influenced by the variety and the degree of matureness, as shown in **Table 2.1**. Carbohydrates make up 54.9% of the weight of fresh date fruit, 42.4% water and the remainder comprising at about 7.5% dietary fibre, 1.5% protein, 1.1% ash and 0.1% fat. Consideration of these properties is essential for sorting, grading and handling of the products (Khan et al., 2008).

Table 2.1: Chemical composition of fresh and dried dates, based on an average of 10 varieties of dates (Al-Farsi and Lee, 2008a).

Chemical composition (g/100g)	Fresh	Dried
Moisture	34.9	7.4
Fibre	7.5	8.0
Protein	1.5	2.1
Fat	0.1	0.3
Ash	1.1	1.6
Carbohydrates	54.9	80.6

2.1.1.1 Carbohydrate

Carbohydrates are the most abundant chemical constituent of dates, consisting primarily of 70-80% reducing sugars like glucose and fructose (Tang et al., 2013) together with non-reducing sugars like sucrose and a small amount of starch (Al-shahib and Marshall, 2003). The carbohydrate content of dates is one of the most important industrial characteristics for both fresh and dry dates (Fadel, 2008) and depends on the water content, cultivar and stage of ripening (Aleid et al., 2000). The primary sugar in fresh date fruit is glucose, whereas dry date cultivars may contain a comparatively high proportion of sucrose (Ghnimi et al., 2017). Date fruit can be categorised into three different groups according to sugar type: 1) invert sugar that contains glucose, 2) the mixed sugar consisting of glucose and fructose and 3) cane sugar which contains sucrose as the main sugar (Ghnimi et al., 2017).

Based on the stage of ripening, the amount of carbohydrates in date fruit increases systematically with the four ripening stages, from *Kimri* through *Khalal* and *Rutab* to *Tamr* (Ahmed et al., 1995b). This is because the reduction of the moisture content of the date through the stages of ripening causes an increase in the concentration of carbohydrate (Al-shahib and Marshall, 2003). Within the first stage of ripening, the *Hababaok* stage, no soluble sugars are detected (Eltayeb et al., 1999). However, a rapid increase in reducing sugars occurs at the *Kimri* stage with an increase in sucrose content during the *Khalal* stage. Several researchers have investigated the overall sugar content in different varieties of date fruit at the *Tamr* stage they found that the highest overall sugar content was 87.5% in the *Khanizi* variety followed by the *Deglet Nour* 74.1% and *Madjool* 66.4% while the *Allig* variety contained only 53.2% (Ahmed et al., 1995b; Al-Farsi et al., 2005b; Rahman et al., 2005; Ismail et al., 2006; Sahari et al., 2007; Elleuch et al., 2008; Khan et al., 2008; Chaira et al., 2009; Rock et al., 2009; Guizani et al., 2010).

2.1.1.2 Dietary fibre

Dietary fibre (DF) may be defined as the indigestible dietary components of the plant materials (Vayalil, 2012). These components are divided into soluble substances (e.g. hydrocolloids and pectin) and insoluble substance (e.g. hemicellulose, lignin and cellulose). Date fruit contains high amount of dietary fibre: 7.5 g/100 g in fresh dates and 8.0 g/100 g in dried dates, the majority of which is insoluble dietary fibre at about 5.8 g/100 g in fresh dates and 5.7 g/100 g in dried dates (Al-Farsi and Lee 2008a). Dates can be a direct supply of dietary fibre within the diet, because 100 g of date provides 32% of the recommended nutrient intake fibre intake, which is 25 g/day (Marlett et al., 2002; Al-Shahib and Marshall, 2003; Manickavasagan et al., 2012).

The content of dietary fibre in dried dates is higher than fresh dates and it depends on the variety and the stage of maturation, with higher fibre content within the early stage of ripening (Shafiei et al., 2010). In the ripening process, insoluble polymers are broken down into smaller soluble molecules by pectinase and polysaccharide enzymes present within the fruit. This is because all the hemicellulose, pectin, lignin and polysaccharide contents decrease as the dates ripen. The polysaccharide, hemicellulose, and lignin contents of date fruit have been found to be 1.5, 1.2, and 2%, respectively (Ashraf and Hamidi-Esfahani, 2011).

2.1.1.3 Fat

Date palm fruit contains a small quantity of fat, which is located mostly within the skin, which is reduced as the fruit ripens. In the *Kimri* stage, the fat content is 0.5% and it is reduced to 0.1% at the *Tamr* stage (Hui, 2006). The function of fat in date fruit is to protect the fruit (Al-shahib and Marshall, 2003; Biglari, 2009). There are eight fatty acids in date palm fruit, however, in very low concentrations. Al-shahib and Marshall (2003) reported that the main unsaturated fatty acid in date fruit was oleic acid, while the rest consist of myristic, palmitic, lauric, pelargonic, arachidic, capric, linoleic and caprylic

acid.

2.1.1.4 Proteins

Protein is present in date palm fruit in small amounts, varying from 2.3% to 5.6%; the average protein content of fresh dates is 1.5 g/100 g and 2.1 g/100 g for dried fruit (Al-Farsi and Lee 2008a). Assirey (2015) investigated different date varieties and found no significant difference in protein content across 10 cultivars: *Ajwa, Anabarah, Burni, Khodari, Shalaby, Sukkari, Labanah, Mabroom, Suqaey* and *Safawy*. The lowest protein content was about 1.7 g/100 g for the *Mabroom* variety and the highest protein content was for the *Shalaby* variety at about 4.7 g/100 g. Based on these findings, the *Shalaby* variety from the KSA has been selected as the sample date for this study. The protein content is relatively high in comparison to other fruits, such as oranges, apple, grapes, and bananas that contain 0.7%, 0.3%, 1.0% and 1.0% protein, respectively (Pennington and Douglass, 2005).

Although date palm fruit contains low amounts of protein, it can nevertheless contribute to the human diet by offering good quality essential amino acids (Al-Hooti et al., 1997). They provide most of the essential amino acids which cannot be made by the body (valine, phenylalanine, tryptophan, methionine, leucine, isoleucine, lysine and threonine), making them an important source of essential amino acids for people living in regions where other sources are limited (Al-Farsi and Lee, 2008a). However, they are poor in sulphur-containing amino acids, such as cysteine and methionine (Siddiq et al., 2014).

There are 17 different amino acids present in date palm fruit proteins, most of which are not found in other popular fruits (**Table 2.2**) for example, glycine, proline, alanine, histidine, arginine, serine and glutamic and aspartic acids are exclusively found in dates**.** According to Ishurd et al., (2004) and Tang et al., (2013), high concentrations of leucine, lysine, serine, alanine and glutamic and aspartic acid are found in the *kimri* stage, while aspartic acid, glutamic acid, proline, leucine, glycine, and lysine are most plentiful during

the ripening stages. Amino acid content increases in dried species, mainly because of water reduction (Auda et al., 1976).

Table 2.2: Amino acid content in fresh and dried dates (Al-Farsi and Lee, 2008a) cited in Siddiq et al., (2014).

Amino acid	Fresh dates (mg/100 g)	Dried dates (mg/100 g)
Alanine	30	133
Arginine	34	148
Aspartic acid	59	309
Cysteine	13	67
Glutamic acid	100	382
Glycine	42	268
Histidine	0.1	46
Isoleucine	4	55
Leucine	41	242
Lysine	42	154
Methionine	4	62
Phenylalanine	25	67
Proline	36	148
Serine	29	128
Threonine	23	95
Tryptophan	7	92
Tyrosine	15	156

2.1.1.5 Minerals

AL Juhaimi et al., (2014) reported that date fruits are a great source of numerous minerals that are significant for metabolism in human cells. 100 g of date fruit contains 362 mg of copper, 696 mg of potassium, 90 mg of magnesium, which is important for bone growth, and 90 mg of iron, which can correct iron deficiencies and anaemia (Al-Shahib and Marshall 2003). Potassium helps to regulate heart rate and blood pressure and is essential for muscle contractions. According to USDA (2007) 100 g of dates contains a mean of 0.8 µg selenium, which may be prevent cancer and stimulate the immune system and 0.3 µg of copper that is required for the production of red blood cells. Consumption of 100 g of date fruit may offer over 15% of the Reference Nutrient Intake (RNI) of potassium, selenium, magnesium and copper for adults (Al-Farsi and Lee, 2008a). According to El Hadrami and Al-Khayri (2012), the fluorine in date fruit ranges from 0.1 mg/100 g to 0.2 mg/100 g which may be helpful in protection against decay.

2.1.1.6 Vitamins

Dates contain vitamins such as thiamin, riboflavin, biotin, vitamin C and B-complex vitamin (B1, B2, B3, B5, B6, B9 (folic acid)) and vitamin K (Al-Farsi and Lee, 2008a). The concentrations of vitamins B3, B5, B6, and B9 in date fruit are higher than in some common fruits, such as orange, berries and apples. 100 g of date fruit provides between 1.2 and 1.6 mg of vitamin B3 (Siddiq et al., 2014). Dates also contain small amounts of vitamins C, B1 and B2 (Al-Shahib and Marshall, 2003). In the mature stages of date fruit vitamins B1, B3, B5 and B6 are very high while vitamins B2, B9 and B12 have been detected in unripe fruit. Although the amount of vitamin C is very low, it is still higher than in figs, raisins, plums, and apricots (Siddiq et al., 2014). Aslam et al., (2013) reported that the chemical analysis of water-soluble vitamins showed a significant variation among the various cultivars and in the developing stages of date fruit.

2.1.1.7 Anti-oxidants

Anti-oxidants (oxidation inhibitors) are a group of materials that decrease oxidative damage. Vaya and Aviram (2001) defined an anti-oxidant as “any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents the oxidation of substrate”. Thus, anti-oxidants in consumed food have been found to act as defensive and protective agents against oxidative species in the human body. Anti-oxidants present in plants also protect the plants themselves by inhibiting oxidation products which otherwise lead to reduced nutritional value and sensory quality of the plant.

Even though oxygen is a substantial element of life, it can create damaging by-products during normal cellular metabolism. Gerschman et al., (1954) attributed the damaging effects of O_2 to the formation of oxygen radicals. This hypothesis was popularised and converted into the “superoxide theory of O_2 toxicity”. In its simplest form, the superoxide theory states that O_2 toxicity is due to an excess creation of O_2 and that the superoxide

dismutase (SOD) enzyme is essential for the catalytic removal of the superoxide free radicals (Halliwell, 1996).

Anti-oxidants can be classified into two main categories, primary or chain-breaking anti-oxidants and secondary or preventative anti-oxidants. Primary anti-oxidants intercept and stabilise free radicals by donating active hydrogen atoms. Secondary anti-oxidants prevent the formation of additional free radicals by decomposing unstable hydroperoxides into a stable product (**Figure 2.1**). Anti-oxidants can be divided into two main classes according to their solubility; 1) hydrophilic anti-oxidants (water-soluble) such as most of the ascorbic acid and phenolic compounds and 2) lipophilic anti-oxidants (fat-soluble), such as vitamin E and carotenoids (**Figure 2.2**) (Namki, 1990).

Javanmardi et al., (2003) indicated that the phytochemicals from fruits possess significant anti-oxidant capacities that may be associated with lower incidence and lower mortality rates of degenerative diseases in humans. The interest in anti-oxidants has been increasing because of their high capacity to scavenge free radicals implicated in different diseases (Silva et al., 2007). Fruits and vegetables contain abundant different naturally occurring anti-oxidant components, such as ascorbic acid (vitamin C), which is a water-soluble anti-oxidant (Naidu, 2003), vitamin E, which is a fat-soluble vitamin (Herrera and Barbas, 2001; Packer et al., 2001), and fat-soluble β-carotene (vitamin A) (Albanes, 1999). Phenolic compounds are also capable of counteracting the damaging effects of oxidation (Jacob et al., 2014).

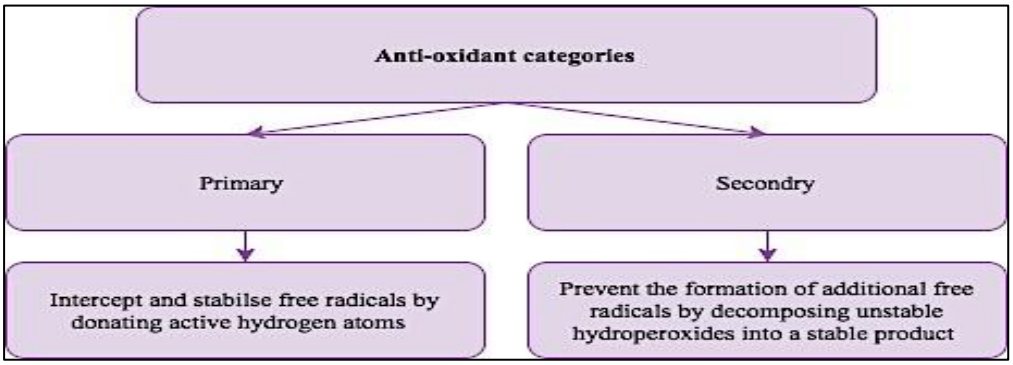


Figure 2.1: Anti-oxidant categories (Namki, 1990).

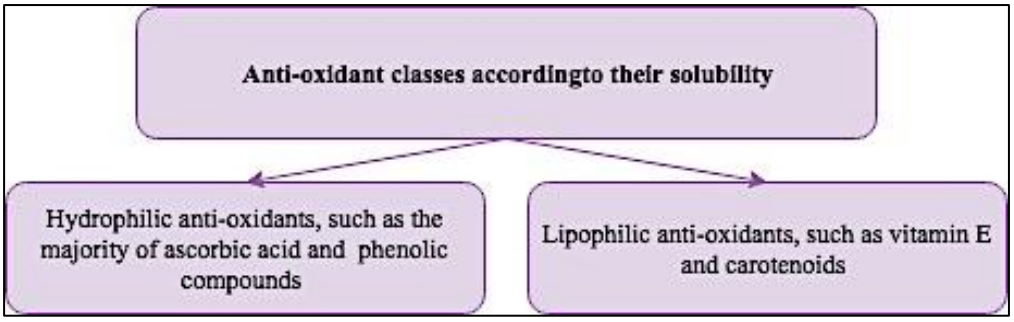


Figure 2.2: Anti-oxidant classes according to their solubility (Namki, 1990).

Anti-oxidants of date palm fruit

The anti-oxidant power of date fruit has been published widely in the scientific literature. The types of dates studied, the different methods used for analysis and the respective authors are depicted in **Table 2.3**.

Table 2.3: Anti-oxidant activity of date palm fruit

Stage of ripening	Method	Values	References
Tamr	ORAC	Ranged from 23.87 to 38.95 µMol Trolox of equivalent/100 g DW	Wu et al., (2004a)
Fresh and sun-dried dates at Tamr stage	ORAC	Ranged from 11.687 to 20.604 µMol Trolox of equivalent/100 g DW in fresh dates. Ranged from 8.212 to 12.543 µMol Trolox of equivalent/100 g DW in sun-dried dates.	Al-Farsi et al., (2005a, 2005b)
Tamr	DPPH	Ranged from 0.08 to 0.22 values of anti-radical efficiency.	Mansouri et al., (2005)

Different stages of ripening. First stage Hababouk, Fourth stage Rutab and Fifth stage Tamr	FRAP	At Hababouk 5.71 mM/100 g fresh weight At Rutab 1.2 mM /100 g fresh weight At Tamr 0.94 mM /100 g fresh weight	Allaith (2008)
Tamr	ABTS	Ranged from 22.83 to 500.33 μMol Trolox of equivalent/100 g DW.	Biglari et al., (2008)
	FRAP	Ranged from 11.65 to 387.34 μMol /100 g DW	
Tamr USA dates KSA dates	ABTS	421.03 mg/100g of USA dates: 389.82 mg/100 g in KSA dates	Al-Turki (2008)
	DPPH	313.24 mg/100 g in USA dates 530.92 mg/100 g in KSA dates	

Most data available concerning anti-oxidants in dates is presented using the ORAC, DPPH and FRAP methods. As can be seen in **Table 2.3** the highest anti-oxidant content determined by using the ORAC method ranged from (23.87 to 38.95 μMol Trolox of equivalent/100 g DW) (Wu et al., 2004b). While the highest content determined by DPPH is 530.92 mg/100 g (Al-Turki, 2008). The table also presents the highest content determined by using FRAP which ranged from 11.65 to 387.34 μMol /100 g DW) (Biglari et al., 2008). The difference in anti-oxidant activity levels perceived can be due to the location where the dates were grown, environmental factors, different cultivars, the extraction methods used and the instrument of analysis and the stage of fruit ripening. A sharp reduction in anti-oxidant activity was found to be associated with the fruit ripening (Allaith, 2008).

It is worth mentioning that the consumption of date fruits at the *Tamr* stage of ripening also provides a total anti-oxidant value equivalent to several common fruits, such as oranges, brussels sprouts and sweet cherries (Blomhoff, 2005). Guo et al., (2003) claimed

dates have the second highest anti-oxidant value after hawthorn fruit amongst 28 fruits generally consumed in China. The anti-oxidant properties of dates as with those of other fruits differ depend on the content of carotenoids, phenolic components, vitamins E and C and flavonoids (Al-Farsi et al., 2005b; Guo et al., 2003; Mansouri et al., 2005; Saura-Calixto and Goni, 2006). It was concluded that phenolic compounds in dates are the main contributors of anti-oxidant activity (Allaith, 2008). The average phenolic content of three varieties of fresh dates is (193.7 mg/100 g) while the average phenolic content of 10 varieties of dried dates is (239.5 mg/100 g) Al-Farsi and Lee (2008a).

Anti-oxidant components are not only critical to the nutritional and functional properties of date fruits (taste, texture, colour, lipid resistance to oxidation) but they can also have many health benefits, such as anti-cancer (Ishurd et al., 2004) and antiviral activities (Al-Farsi et al., 2005a).

2.1.1.8 Anti-nutritional factors

Anti-nutritional factors are compounds, which reduce the nutrient utilization of plants or plant products consumed as human foods or animal feed (Gemede and Ratta, 2014). High consumption of some fruits and vegetables can be hazardous as they might contain anti-nutritional factors. For instance, tannin, phytic acid, saponin and oxalic acid are anti-nutritional factors that have adverse effects on health through the inhibition of protein digestion (Larsson et al., 1996).

Emire et al., (2013) classified the anti-nutritional factors into two main categories which are:

1- Proteins, for instance protease inhibitors and lectins, which are sensitive to normal processing temperatures.

2- Haemaglutinins, cyanogenic glycosides, saponins, tannins, oestrogens, gossypol from *Gossypium* spp, amino acid analogues, alkaloids, plant enzymes and enzyme inhibitors.

Based on literature (Shaba et al., 2015; Nadeem et al., 2011; Elinge et al., 2012; Agbaire, 2012) the most common anti-nutritional factors in date palm fruit are tannins, oxalate and phytate. Based on this information, these anti-nutritional factors were selected to be determined in date fruit protein extract. The published values for date palm fruit are listed in the table below.

Table 2.4: Anti-nutritional factors of date palm fruit

Stage of ripening	Anti-nutritional factors	mg/100 g DW	References
Tamr	Tannin	Ranged from 0.22 to 0.87	Nadeem et al., (2011)
	Oxalate	Ranged from 0.30 to 0.77	
	Phytate	Ranged from 3.63 to 6.49	
	Oxalate	7.57	Shaba et al., (2015)
	Tannin	5.25	

Tannins

Tannin is an astringent, bitter polyphenolic plant compound that either binds or precipitates proteins and several other organic compounds, including amino acids and alkaloids (Redden et al., 2005). The molecular weights of tannin ranges from 500 to 3000 (Muzquiz et al., 2000) and they are heat stable. Tannins are known to inhibit the activities of chemo-trypsin, lipase and amylase and to interfere with dietary iron absorption (Mello, 2000).

Consumption of food containing high doses of tannin can cause bowel irritation, liver damage, gastrointestinal pain, kidney and stomach irritation as it chelates minerals, such as iron leading to cause anaemia (McGee, 2004; Karamac, 2009). It also binds to protein and reduce their nutritional value (El-Shemy et al., 2000). Tannins decrease protein digestibility in humans and animals by inhibiting digestive enzymes by forming protein tannin complexes and is responsible for decreasing the growth rate in experimental animals (Kyriazakis and Whittenmore, 2006).

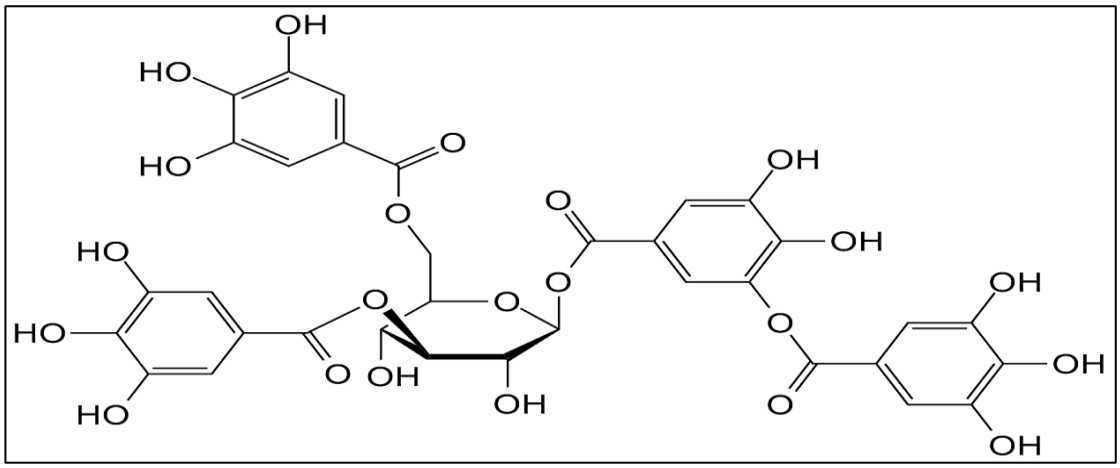


Figure 2.3: Chemical structure for tannin.

Oxalate

Strong bonds are formed between oxalic acid and several minerals, such as potassium, magnesium, calcium and sodium forming oxalate salts. Some oxalate salts are soluble in water, such as potassium and sodium oxalates, and some of them are insoluble, such as calcium oxalate. Calcium oxalate is insoluble at a neutral or alkaline pH, but easily dissolves in acid (Liener, 1994). The insoluble calcium oxalate has the tendency to precipitate in the kidneys or in the urinary tract. When the levels of this salt are high enough, it will form sharp-edged calcium oxalate crystals. These crystals are responsible for the formation of kidney stones (Gemede and Ratta, 2014). Agbaire (2012) also reported that oxalates cause irritation and swelling in the month and throat.

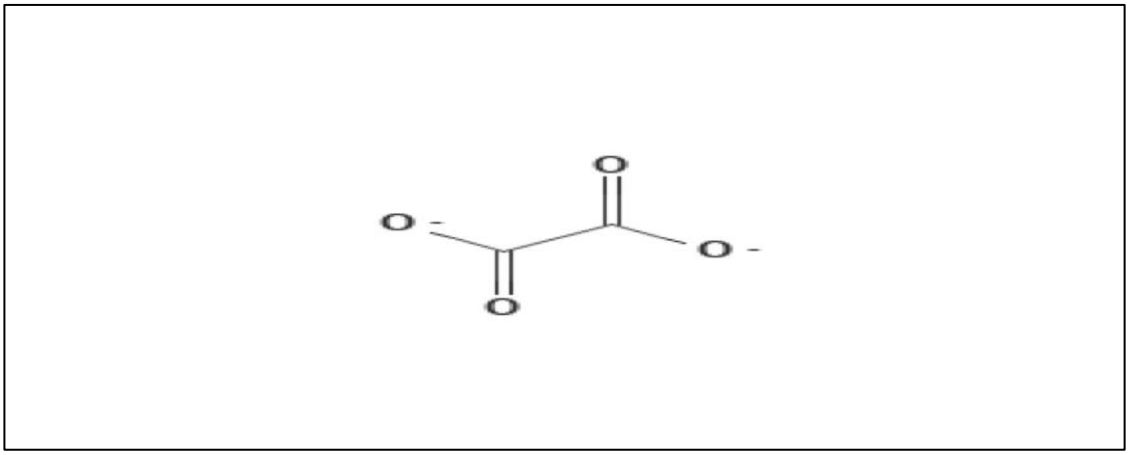


Figure 2.4: Chemical structure for oxalate.

Phytate

This is the main component of plant storage organs where it serves as a phosphate source for germination and growth (Aberoumond, 2009). Phytate is the storage form of phosphorus and is abundant in foods having high content of fibre (Nadeem et al., 2011). Phytate binds minerals in the gastrointestinal tract, making the dietary minerals unavailable for absorption and utilization by the body (Rathod, and Valvi, 2011). It also forms calcium phytate complexes that inhibit the absorption of Fe, Zn and decreases calcium bioavailability (Rathod, and Valvi, 2011). Consumption of food with high levels of phytate will lead to mineral deficiency and pellagra (Ali et al., 2010). There is some concern about phytate intake in some regions of the world where cereal protein is the main and prevalent dietary factor (Mueller, 2001).

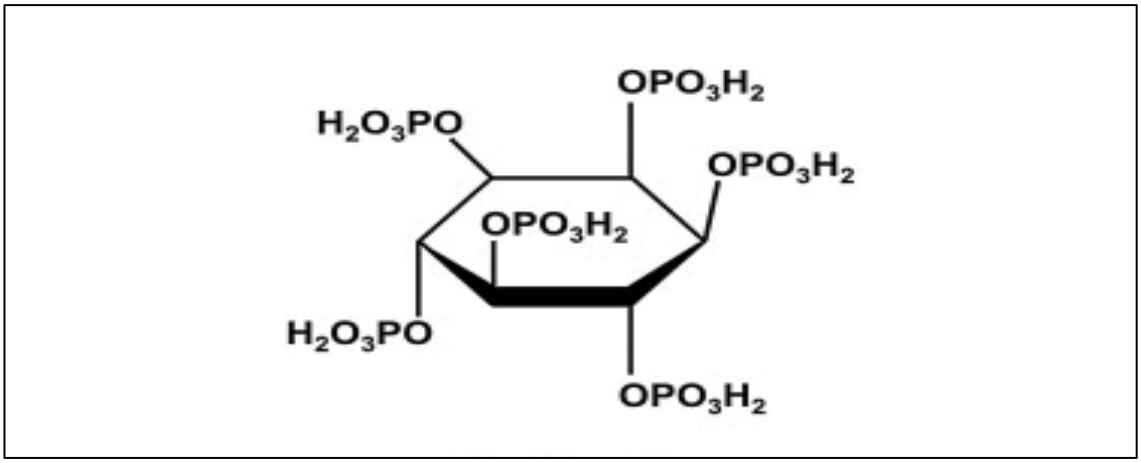


Figure 2.5: Chemical structure for phytate.

2.2 Chemicals and methods

Chemicals

Table 2.5: Chemicals obtained from (Sigma Aldrich, UK).

Chemicals obtained from Sigma Aldrich, UK	Catalogue number
2,2-Diphenyl-1-picrylhydrazyl ($C_{18}H_{12}N_5O_6$) (DPPH)	1898-66-4
Ascorbic acid ($C_6H_8O_6$)	50-81-7
Methanol 95% (CH_3OH)	67-56-1
Iron(II) sulphate heptahydrate ($FeSO_4 \times 7H_2O$)	7782-63-0
Iron(III) chloride ($FeCl_3$)	7705-08-0
Glacial acetic acid (CH_3COOH)	A6283
2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ)	3682-35-7
Sodium acetate ($C_2H_3NaO_2$)	127-09-3
Sodium phytate ($C_6H_6Na_{12}O_{24}P_6$)	P8810
Thioglycolic acid ($C_2H_4O_2S$)	5280566
NaOH	S8045
Nitric acid (HNO_3)	7697-37-2
Phosphate buffered saline	P4417
Pepsin	P7000
Pancreatin,	8049-47-6
Sodium azide (NaN_3)	26628-22-8
Trichloroa-cetic acid ($C_2HCl_3O_2$)	76-03-9
Anthrone ($C_{14}H_{10}O$)	90-44-8
D- (+) Glucose analytical standard	50-99-7

Table 2.6: Chemicals obtained from (Thermo Fisher Scientific, UK).

Chemicals obtained from Thermo Fisher Scientific, UK	Catalogue number
Sodium tungstate (Na_2WO_4)	10144740
Phosphomolybdic acid ($H_3[PMo_3O_{10})_4]\cdot H_2O$	10660175
Sodium carbonate (Na_2CO_3)	10264540
Tannic acid **(**$C_{76}H_{52}O_{46}$)	10627761
Potassium permanganate ($KMnO_4$)	10246360
Sulphuric acid (H_2SO_4)	10294300
(Ammonium iron (II) sulphate hexahydrate **(**$(NH_4)_2Fe(SO_4)_2 \times 6H_2O$	10105183
2,2'-bipyridine	11492438
HCl	10488020
Whatman No.1	11322815
Phosphoric acid (H_3PO_4)	7664-38-2

Methods

2.2.1 Chemical composition of date palm fruit protein extract

2.2.1.1 Measurement of moisture and total solids content

Moisture in date palm fruit protein extract was determined by using the AOAC method No. 925.10 (AOAC, 1995). (1) An aluminium dish was cleaned and dried at 105ºC and weighed. (2) 2 g of dried date sample was weighed into the dishes and then heated in an air oven overnight at 105˚C (3). The dish was cooled and weighed.

The percentage of moisture was calculated by using the following formula:

$$Percentage\ of\ moisture\ = \frac{(\text{weight of 3}) - (\text{weight of 1})}{(\text{weight of 2})} * 100$$

The percentage of the total solids was calculated by using the following formula:

Percentage of total solids = 100 – (% moisture).

2.2.1.2 Total ash

The ash content was measured based on the AOAC method No. 923.03(AOAC, 1995). (1) A crucible was cleaned and dried at 105ºC and weighed. 4 g of dried date sample were weighed into the pre-weighed crucible. (2) The crucible containing the sample was heated at 550˚C in a muffle furnace for 5-6 hours. The crucible was weighed after reaching room temperature (RT).

The percentage of total ash was calculated based on the following formula:

Weight of ash = (2)-(1)

$$Percentage\ of\ total\ ash\ = \frac{\text{Weight of ash}}{\text{Weight of sample}} * 100$$

2.2.1.3 Crude Fibre

Crude fibre was determined in a sample based on the AOAC method (AOAC, 1995, method No. 962.09). 2 g of dried date sample were weighed into a 1000 ml beaker. 200 ml 1.25% (v/v) of sulphuric acid was added carefully to the beaker. The sample was digested by boiling in water bath for 30 min with continuous agitation to prevent burning

on of the sample on the walls of the beaker. The boiled solutions were filtered using vacuum filtration apparatus. The residue was washed three times with hot water and transferred to a new 1000 ml beaker. The digestion was repeated but with 200 ml 1.25% (w/v) NaOH instead of sulphuric acid.

After filtration of the samples the residue was washed three times with 1% (v/v) HCl to remove any alkali and three times with hot water to remove any trace of acid. The residue was transferred to a clean, pre-weighed crucible and dried for 24 h at 105˚C in an air oven. The dried residue was burned for 5-6 hours at 550 °C in a muffle furnace. The percentage of crude fibre was calculated by using the following formula:

$$Percentage\ of\ crude\ fibre = \frac{\text{Weight of residue (g)} - \text{Weight of ash (g)}}{\text{Weight of sample (g)}} * 100$$

2.2.1.4 Crude fat

Crude fat was carried out by Sciantec Analytical Services Ltd, UK, using the following method: The fat was obtained by the continuous extraction of the sample with warm light petroleum ether - boiling range 40-60°C. The solvent was removed by evaporation and the dry fat was weighed. This treatment does not extract oxidised oils, phospholipids or fatty acids combined as soaps.

2.2.1.5 Carbohydrates

To measure the content of carbohydrate present in the DFPE sample the anthrone method was used, as described by Hedge and Hofreiter (1962) with some modifications.

Principle: Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In a hot acidic medium, glucose is dehydrated to hydroxymethyl furfural. This compound forms a green coloured product with anthrone, which has an absorption maximum at 630 nm.

Preparation of reagents

1) 200 mg anthrone was dissolved in 100 ml of ice-cold 95% H_2SO_4. It was prepared fresh before use and was protected from light in a dark bottle and used within 10 h, as recommended. 2) 100 mg of D-(+) glucose was dissolved in 100 ml H_2O and the working standard used was 10 ml of stock diluted to 100 ml with distilled water. The solution was refrigerated.

Determination method

100 mg of DPFE was weighed into a boiling tube. The sample was hydrolysed by keeping it in a boiling water bath for 3 hours with 5 mL of 2.5 N HCl and cooled to room temperature. The sample was neutralised with solid sodium carbonate until the effervescence ceased. The volume was then made up to 100 ml and centrifuged. The supernatant was collected and 0.5 and 1 ml aliquots taken for analysis.

The standard solution was prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard. The solution containing no protein sample served as the blank. The volume was made up to 1 ml in all the tubes including the sample tubes by adding dH_2O. The contents of all the tubes were cooled on ice before adding ice-cold anthrone reagent. Then 4 ml of anthrone reagent was added carefully to each tube of standard solution and to the test tube. The tubes were heated for eight min in a boiling water bath. They were cooled rapidly and the absorbance of the green to dark green coloured solution was measured at 630 nm.

A standard graph was drawn by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis. From the graph the amount of carbohydrate present in the sample tube was calculated.

Calculation

$$\text{Amount of carbohydrate present in 100 mg of the sample} = \frac{\text{mg of glucose}}{\text{ml of test sample}} * 100$$

2.2.2 Nutritional analysis of date palm fruit protein extract

2.2.2.1 Determination of total essential and non-essential amino acids content

Amino acid analysis was performed by Sciantec Analytical Services Ltd., UK by using the following method:

The sample was oxidised with hydrogen peroxide/formic acid/phenol solution, which converts any methionine to methionine-sulphone and any cysteine to cysteic acid, as some of the cysteine and methionine would otherwise be lost upon hydrolysis. The excess of oxidation reagent was decomposed with sodium meta-bisulphite. The oxidised sample was hydrolysed with 6 M HCl for 24 h at 110°C. The hydrolysate was adjusted to pH 2.20 and the amino acids were separated by cation exchange chromatography to separate polar molecules in the liquid phase based on their affinity to the cation exchange solid phase and measured by post column reaction with ninhydrin reagent using photometric detection at 570 nm. The test was performed by Sciantec analytical Services Ltd.

2.2.2.2 *In vitro* protein digestibility (IVPD)

The digestibility of protein is a significant factor, to estimate the availability of protein for absorption after digestion, which reflects the efficiency of protein utilisation in the diet (World Health Organization, 2007). It is also an indication of the presence of anti-nutritional factors, such as protease inhibitors. The *in vitro* protein digestibility (IVPD) assay is a widely used method to determine the digestibility parameter as the amino acid score (AAS) does not consider whether the protein is digestible or not (Mokrane et al., 2010). The protein digestibility-corrected amino acid score (PDCAAS) is a recognised and approved method for evaluating protein quality, considering the AAS and the digestibility parameter of the food matrix. This parameter derives from the AAS and is corrected based on the digestibility assay of the protein (World Health Organization, 2007).

Method for determination of (IVPD) of date fruit protein extract

The *in vitro* protein digestibility was evaluated according to the method described by Almeida et al., (2015) with modifications. Samples of 250 mg DFPE and SPI (positive control) samples or 250 µl of deionized water (the blank) were respectively suspended in 15 ml of 0.1 M HCl containing 1.5 mg/ml pepsin and incubated in a water bath at 37 °C for 3 hours. The pepsin hydrolysis ceased after neutralization with the addition of 7.5 ml of 0.5 M of NaOH. Pancreatic digestion was then initiated with the addition of 10 ml of 0.2 M phosphate buffer (pH 8.0) containing 10 mg of pancreatin. 1 ml of 0.005 M sodium azide was then added to prevent microbial growth and incubated overnight at 37 °C. After the pancreatic hydrolysis, 1 ml of 10 g/100 ml of TCA was added, followed by centrifuging at 5000 rpm at RT for 20 min using (Beckman Coulter, Avanti J-26 XP centrifuge with a JA 25.50 rotor, Beckman Coulter, USA). The supernatant was collected, and the total protein content was determined based on the nitrogen content, using Kjeldahl AOAC method 930.29 (AOAC, 1995).

The IVPD values were calculated based on the following equation:

$$Percentage\ of\ true\ digestibility = \frac{(\mathrm{Ns} - \mathrm{Nb})}{(\mathrm{Ns})} * 100,$$

where Ns and Nb represent the nitrogen content in the sample and in the blank, respectively.

2.2.2.3 Amino acid score (AAS) and protein digestibility-corrected amino acid score (PDCAAS)

The AAS was calculated based on the calculation of Hughes et al., (2011) using the following equation:

$$Amino\ acid\ score = \frac{\text{mg of LAA in 1.0 g of test protein}}{\text{mg of the same LAA in 1.0 g of reference protein}} * 100$$

The PDCAAS was calculated using the FAO/WHO prescribed equation:

$$Percentage\ of\ PDCAAS = \frac{\text{Amino acid score of the most limiting AA}}{\%\ \text{true digestibility}} * 100$$

where LAA is the most limiting amino acid in test proteins.

2.2.2.4 Mineral analysis

Mineral content was measured by using atomic absorption spectrophotometry by Mr. Hugh Barras (Technician at the School of Engineering and Physical Science EPS, Heriot Watt University). The concentration of the sample was (91.75 mg/ml) and that sample was diluted with water. Concentrated HNO_3 was added to the sample in a volumetric flask and left to digest in a fume cupboard for 3 hours. Distilled water was added to the sample and the flask was placed into a water bath at 60°C for 2 hours. The extract was filtered through GF/C filter paper in a 50 ml volumetric flask. Measurements were made in triplicate and the mineral content was determined by atomic absorption spectrophotometry.

Principle of the method

The principle of atomic absorption spectrometry (AAS) is based on the principle that different atoms absorb specific wavelengths of electromagnetic radiation. The instrument consists of a light source, atomizer, monochromator and detector. Free atoms (gas) is generated by the atomiser. The free atoms absorb radiation from the light source and monochromator at a specific frequency (García and Báez, 2012). AAS use the absorption of light to measure the concentrations of atoms in the gas phase.

2.2.2.5 Anti-oxidant analysis

Some researchers advise the implementation of several methods to evaluate the anti-oxidative capacity of food components (Erkan et al., 2008; Rzaeizadedeh et al., 2011). On the other hand, other publications reported selection of one or two methods as sufficient depending on the purpose (Zainol et al., 2003; Mylnikov et al., 2005; Thaipong et al., 2006; Vangani and Van Wyk 2013).

The methods used to measure the anti-oxidative activity of compounds are classified as direct and indirect methods based on their inactivation mechanism of free radicals. Direct

methods utilize oxidizable substrates, such lipids to determine the inhibitory potential of an anti-oxidant by subjecting these substrates, to natural or accelerated oxidation conditions and are usually used to measure the anti-oxidant capacity of components in oil containing foods with the aim to prolong shelf life (Zainol et al., 2003). These methods including thiobarbituric acid (TBA) and the ferric thiocyanate (FTC) methods (Zainol et al., 2003). Indirect methods measure the ability of a molecule to reduce a stable, artificial free radical by means of hydrogen donation or electron transfer (Vangani and Van Wyk, 2013). These methods include the oxygen radical absorption capacity (ORAC) (Ou et al., 2002), ferric reducing anti-oxidant power (FRAP) (Guo et al., 2003), diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams et al., 1995) and 2,2-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Leong and Shui, 2002).

The total anti-oxidant content of fresh and dried dates has been reported using indirect methods which are ORAC, FRAP, DPPH and ABTS methods (Al-Turki, 2008). In the present study DPPH and FRAP were selected due to most available data concerning anti-oxidant in dates palm fruit is reported using DPPH and FRAP methods (Mansouri et al., 2005; Allaith 2008; Biglari et al., 2008; Al-Turki 2008).

The principles for the DPPH and FRAP methods are described below.

2.2.2.5.1 Radical Scavenging Effect Assay (DPPH)

In the radical scavenging assay, the anti-oxidants in the sample interact with 2,2-diphenyl-1-picrylhydrazyl (DPPH), and turns into the yellow-coloured, diphenyl-β-picryl hydrazine. The degree of discolouration indicates the radical-scavenging potential of the sample. This procedure was based on that described by Shen et al., (2010) with some modifications. A 0.1 mM solution of 2,2-diphenyl-1-picrylhydrazyl in methanol 95% (v/v) was prepared and 1ml of this solution was added to 3 ml of the DPFE in methanol

solution at different concentrations (10, 20 and 50 mg/ml). The mixtures were vortexed for 30 second and allowed to stand at RT for 30 min.

The absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Novapec II Biochron Ltd., UK).

Ascorbic acid dissolved in methanol at different concentrations (10, 20 and 50 mg/ml) was used to make a standard curve. dH_2O was used as a blank. The DPPH scavenging effect was calculated with the following equation:

$$Radical\ Scavenging\ Effect\ (\%) = \frac{(A_0 - A_1)}{A_0} * 100$$

where, A_0 is the absorbance of the control reaction at 517 nm, and A_1 is the absorbance in presence of the date extract sample at 517 nm.

2.2.2.5.2 Ferric Reducing Anti-oxidant Potential Assay (FRAP)

The procedure used was based on that described by Benzie and Strain (1996), with some modifications.

Acetate buffer 300 mM, pH 3.6 was prepared by dissolving 3.1g of sodium acetate ($C_2H_3NaO_2$) and 16 ml of CH_3COOH in 1 litre of dH_2O. The pH was adjusted to 3.6 pH using NaOH. 10 mM of 2,4,6-Tris (2-pyridyl)-*s*-triazine (TPTZ) was prepared by dissolving 0.321 g of TPTZ in 40 mM HCl. Iron chloride (III) solution (20 mM) was prepared by dissolving 0.324 g in 1 litre dH_2O. The working FRAP reagent was freshly prepared by mixing 2.5 ml TPTZ solution, 2.5 ml Iron chloride (III) solution and 25 ml acetate buffer. 2.85 ml of FRAP reagent was then mixed with 150 µl of the solution of date palm extract at concentration of (20 mg/ml). 20 mg of ascorbic acid was used as a positive control and dH_2O was used as a blank. The mixtures were vortexed for 30 second and allowed to stand at RT for 30 min. The absorbance was measured at 593 nm using a UV-VIS spectrophotometer (Novapec II Biochron Ltd., UK). The absorbance of the samples at 593 nm were compared to the Iron (II) sulphate heptahydrate × $7H_2O$ standard curve and the FRAP values expressed as Iron (II) sulphate heptahydrate equivalents.

2.2.2.6 Anti-nutritional factors analysis

The anti-nutritional factors tannin, phytate and total oxalate contents of DFPE were determined using the following methods.

2.2.2.6.1 Tannin contents

Tannin content was determined by using the Folin-Danis method recommended by Schanderi (1970) with some modifications.

Preparation of the standard solutions

- Folin-Danis reagent was prepared by adding 750 ml dH_2O in a 1000 ml volumetric flask containing 100 g sodium tungstate (Na_2WO_4) and 20 g phosphomolybdic acid $H_3[PMo_3O_{10})_4]H_2O$. After making up the solution, 50 ml of phosphoric acid was added and stirred for 2 hours after which the volume was made up to 1000 ml with dH_2O. The solution was left to stand overnight and then filtered using glass wool.
- Sodium carbonate solution was prepared by dissolving 350 g of sodium carbonate (Na_2CO_3) in 1000 ml dH_2O at 70ºC.
- The solution of standard tannic acid was prepared by dissolving 100 g of tannic acid in dH_2O in a 100 ml volumetric flask. The stock solution was diluted with dH_2O to obtain the working solution of 50 µg/ml of tannic acid.

Method

75 ml dH_2O was added into a 250 ml conical flask containing 500 mg of DFPE sample. The solution was boiled in water bath for 30 min and then centrifuged at 2000 rpm at RT for 20 min. The supernatant was collected and diluted to 100 ml with dH_2O. 5 ml of Folin-Danis reagent 95, 1ml of the sample extract, and 10 ml sodium carbonate solution were added into a 100 ml flask containing 75 ml dH_2O and the volume was made up to100 ml. The mixture was shaken well and the absorbance was measured at 700 nm after incubating for 30 min. For the blank solution dH_2O was used. The standard curve was made using 0-100 µg of tannic acid.

2.2.2.6.2 Phytate content

Phytate content in DFPE was determined by the method described by Haug and Lantzsch (1983) with some modifications.

Preparation of the standard solutions

- The standard sodium phytate stock solution was prepared by dissolving 0.15 g sodium phytate in 100 ml of dH_2O. The standard sodium phytate solution was prepared by diluting the stock solution in a 100 ml volumetric flask in the range of 1.2 to 11.7 ml stock solution and the volume made up with 0.2 N HCl.
- The ferric solution was prepared by dissolving 0.2 g of Ammonium Iron (II) Sulfate Hexahydrate in 100 ml of 2 N HCl and the volume made up to 1000 ml with dH_2O.
- Bipyridine solution was prepared by dissolving 10 g of 2,2-bipyridine and 10 ml thioglycolic acid in dH_2O and the volume was made to 1000 ml.

Method

10 mL 0.2 N HCl solution was added to a 0.06 g of DFPE sample in a test tube and shaked on a bench top shaker for 1 hour. 1 ml of the extracted solution was added into a test tube along with 2 ml ferric solution and covered with a cap. The test tube was heated in a water bath for 30 min then cooled on ice for 15 min. Bipyridine solution 4 ml was added into the test tube and mixed well. After 60 seconds the absorbance of the mixture was measured at 519 nm. The method was calibrated with reference (sodium phytate solution) as a substitute for the sample solution for each set of analyses.

2.2.2.6.3 Total oxalate

Total oxalate was determined by using the method as described by Day and Underwood (1986) with some modifications. 75 ml of 15 N sulphuric acid (H_2SO_4) was added into a conical flask containing 1 g of DFPE powder. The solution was stirred with a magnetic stirrer for 1 h and then filtered using filter paper Whatman No.1. The filtered sample (25

ml) was collected and titrated against 0.1 N of potassium permanganate ($KMnO_4$) solution until a faint pink colour appeared that persisted for at least 30 sec.

The concentration of oxalate in the sample was obtained from the calculation:

1 ml 0.1 N of ($KMnO_4$) = 0.006303 g oxalate (Shaba et al., 2015).

2.3 Results and discussion

Triplicate determinations were performed for the analysis, using freshly prepared DFPE samples. The mean (±) and standard deviation (SD) values were calculated from these triplicates. A p value of (<0.05) was considered statistically significant using the independent samples t-test SPSS version 10 for Windows (SPSS Inc., NY, USA).

2.3.1 Proximate composition of date palm fruit protein extract and date syrup

The proximate composition of date fruit protein extract (DFPE) is shown in **Figure 2.6**

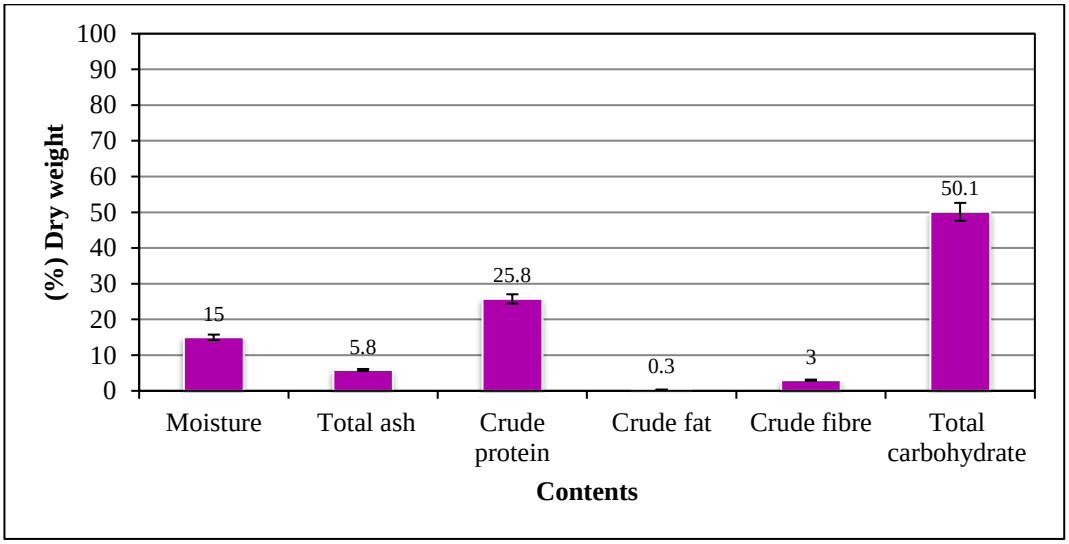


Figure 2.6: Proximate composition of date fruit protein extract powder.

The moisture content of the sample was 15% and 5.8% total ash. The crude protein in the present sample was 25.8%, which was significantly higher than that determined by Al-Hooti et al., (1997) ranging from 2.1 to 6.4% and Assirey (2015) ranging from 1.72 to 4.73 %. To the researcher's knowledge, this finding of such a protein high content from date palm fruit has not been reported before.

The sample also had a very low-fat content at about (0.3%), which is similar to the value of 0.3% for date fruit reported by Sawaya et al., (1983). The fibre content in the sample was 3%, which falls within the range for date fruit reported in the literature ranging from 4 to 8% (Lund et al., 1983; Holland et al., 1991; Spiller, 2001). These differences have been attributed to the analytical techniques adopted. Several studies have reported higher fibre content in date fruit than that found in this study, which could be attributed to the extraction method resulting in the higher protein content of DFPE. Lund et al., (1983) determined the content of fibre by using an enzymatic method and found the content was 6.9% insoluble and 2.3% soluble content.

Carbohydrates were the most prevalent compounds in the sample. As mentioned in (**Section 2.1.1.1**) of this chapter the carbohydrate content of date palm fruit depends on the water content, cultivar and stage of ripening (Aleid et al., 1999; Aleid et al., 2000). In the present study date at *Tamr* stage was used where the carbohydrate content is at its highest level. The overall carbohydrate content was found to be 50.1%, which is lower than that previously reported, where the content in all previous researches ranged from (75.09 to 80.41%) (Al-Farsi et al., 2005b; Akasha et al., 2012; Mohamed et al., 2014; Assirey, 2015). The reason for the reduction of carbohydrate content in the sample in this study could be due the loss of some carbohydrate during the extraction process.

The proximate composition of date syrup is shown in **Figure 2.7**, the highest content in the syrup is carbohydrate at about (71.6%) of which sugars (61.7%) and protein 1.6%.

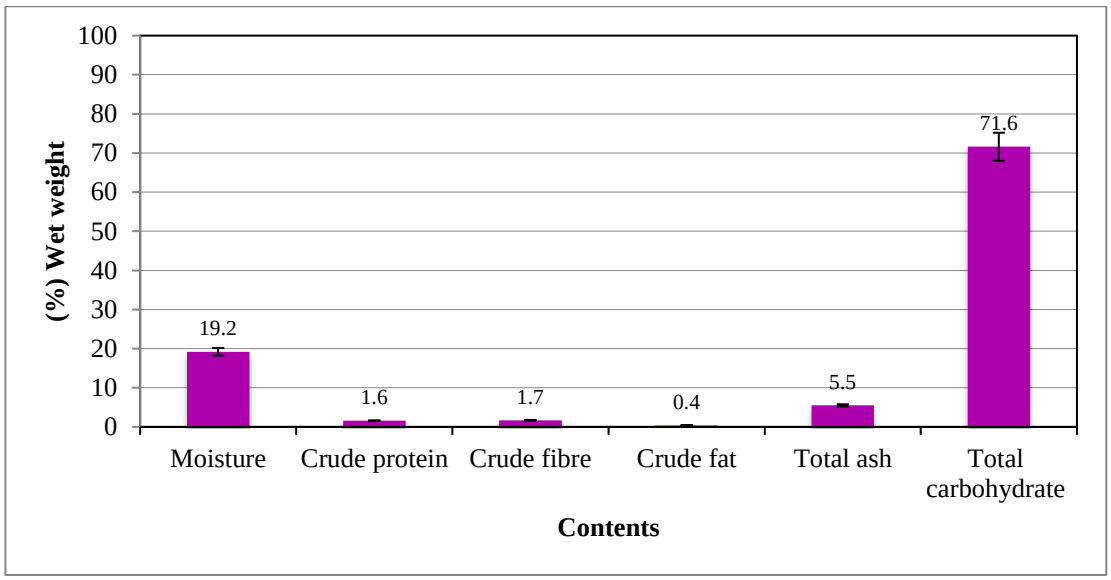


Figure 2.7: Proximate composition of date syrup.

2.3.2 Essential and non-essential amino acids content of DFPE

The amino acid distribution in DFPE is depicted in **Figure 2.8**.

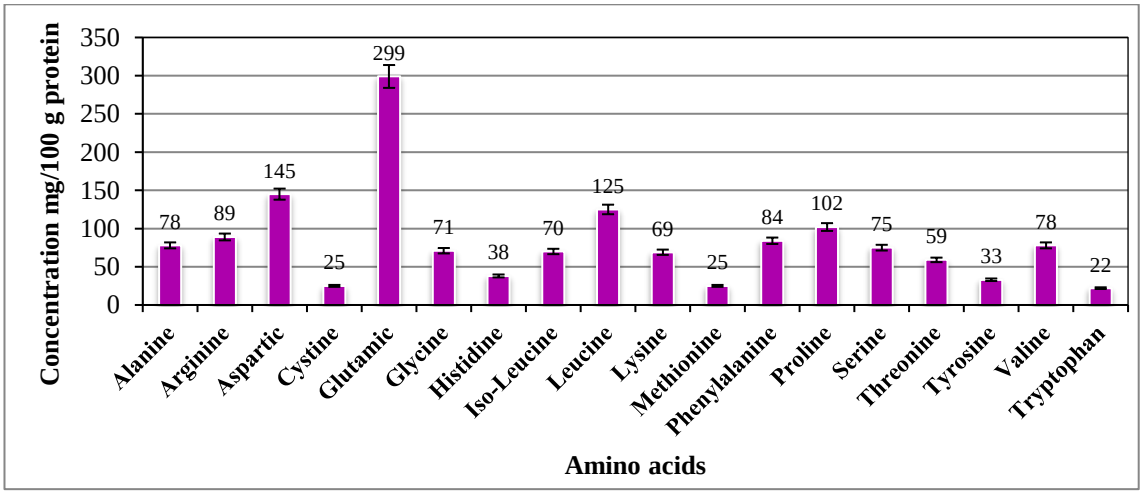


Figure 2.8: Essential and non-essential amino acid of DFPE.

Figure 2.8 shows that DFPE contains all the essential amino acids, which cannot be made by the body (cf. Table 1.2). The highest amino acid content was glutamic acid (299 mg/100 g protein), which was similar to that reported earlier (Assirey, 2015) (158-265 mg/100 g), Tryptophan was the minor amino acid at about (22 mg/100 g protein). This observation was in accordance with those reported by Assirey (2015). The low content of tryptophan 22 mg/100 g, methionine and cysteine 25 mg/100 g could be attributed to the

destruction during acid hydrolysis, which is part of the analytical method for amino acid determination (Salim and Ahmed, 1992) this result confirming the results of Siddiq et al., (2014).

2.3.3 *In vitro* protein digestibility (IVPD) assay for date fruit protein extract

The IVPD result presented in **Figure 2.9** shows that the value for SPI is higher than those reported by Almeida et al., (2015) (55.2%) but similar to that reported by Hughes et al., (2011) of 97.3% for soy protein isolate.

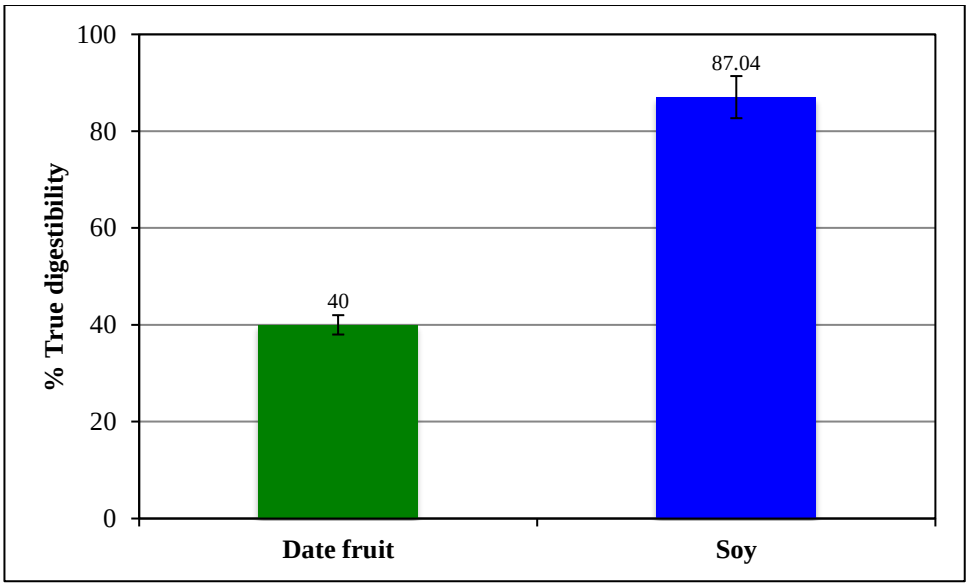


Figure 2.9: Percentage of digestibility of DFPE compared with soy protein isolate.

The digestibility of DFPE is low compared to SPI (40% versus 87.04%) but is comparable to values of (42- 57%) reported by Babatandu et al., (2017) for Amaranthus seed protein extract. The low digestibility could be attributed to the presence of fibre in the DFPE extract. Dégen et al., (2007) reported that the presence of soluble fibre reduced the ileal digestibility of protein in pigs. The reduced digestibility could also be caused by the relatively high level of oxalate and tannin in DFPE (**Table 2.9**). Olawoye and Gbadamosi (2017) demonstrated that reduction in oxalate and tannin content increased the *in vitro* digestibility of Amaranthus seed protein extract.

Here the digestibility of protein extract from date fruit is reported for the first time.

2.3.4 Amino acid score (AAS) and protein digestibility-corrected amino acid score (PDCAAS)

Protein digestibility-corrected amino acid score (PDCAAS) was calculated based on the reference values for human milk suggested by the (World Health Organization, 2007). The value (72%) was compared to that of soy protein isolate (100%), using (methionine) as limiting amino acid in the reference (Umezawa et al., 1993). The limiting amino acid in DFPE was tryptophan and cysteine in soy protein isolate.

Table 2.7: PDCAAS values for DFPE compared to other protein sources.

Protein	% PDCAAS
Date Fruit Protein Extract	72
Soy protein isolate	100
Casein	100
Egg wh0ite	100
Skimmed milk	100

The PDCAAS for DFPE was lower than that of SPI, casein and egg white as reported by FAO/WHO (1989). The PDCAAS value for date protein extract is reported here for the first time.

2.3.5 Mineral analysis

The results of the analyses for minerals in DFPE are presented in **Figure 2.10**.

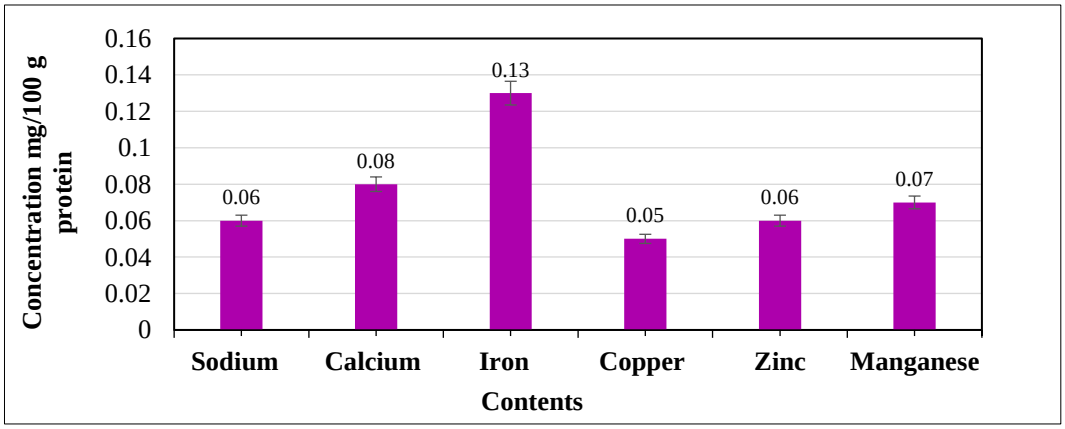


Figure 2.10: The minerals content of date fruit protein extract.

DFPE contains a high level of iron (0.13 mg/100 g), which is essential for red blood cell production (Schoorl et al., 2012). It also contains calcium (0.08 mg/100 g) and magnesium (0.07 mg/100 g) of which calcium is important for metabolism in human cells (Gasim, 1994) and both minerals are essential for healthy bone development. Shaba et al., (2015) found the amount of calcium in date fruit to be between (123-187 mg/g) dry matter, which were higher than the value presented here, the reduction in calcium could have occurred during extraction. The results indicate that DFPE contained considerable amounts of minerals and this correspond to the findings reported by Al-Hooti et al., (19997) and Assirey (2015) in date fruit. These values are three to five times the amounts found in apples, bananas, oranges and grapes (Mohamed et al., 2014).

2.3.6 Anti-oxidant analysis

Radical Scavenging Effect Assay (DPPH)

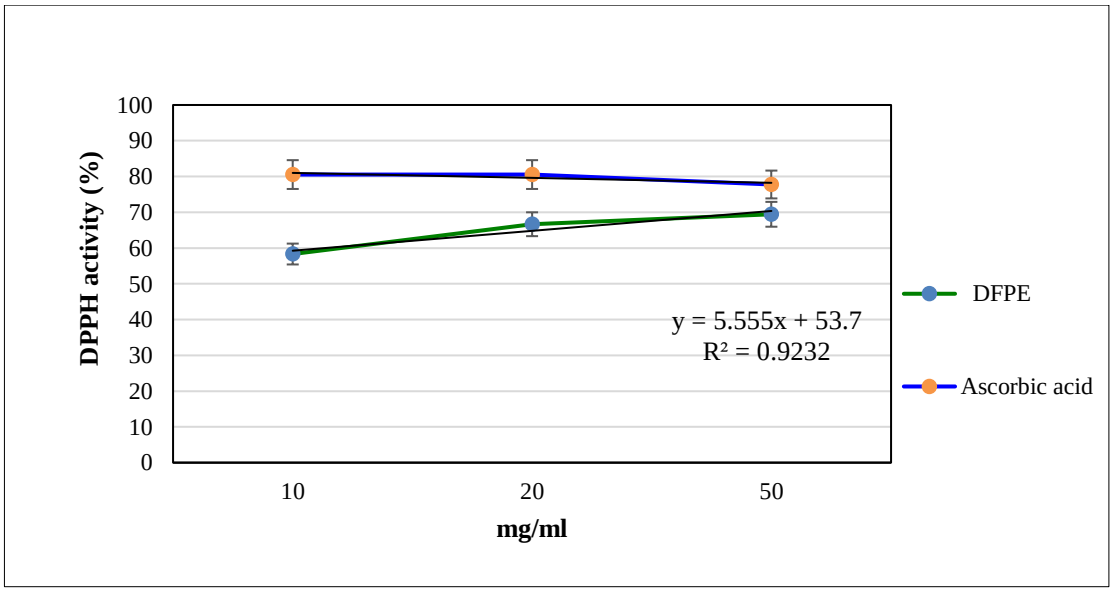


Figure 2.11: DPPH Radical Scavenging activity (%) of DFPE.

In this experiment the date fruit protein extract was found to exhibit a high level of radical scavenging activity, close to that of ascorbic acid. It appears that DFPE has lower anti-oxidant potential compared to the same concentration of ascorbic acid.

The Ferric Reducing Anti-oxidant Power assay (FRAP)

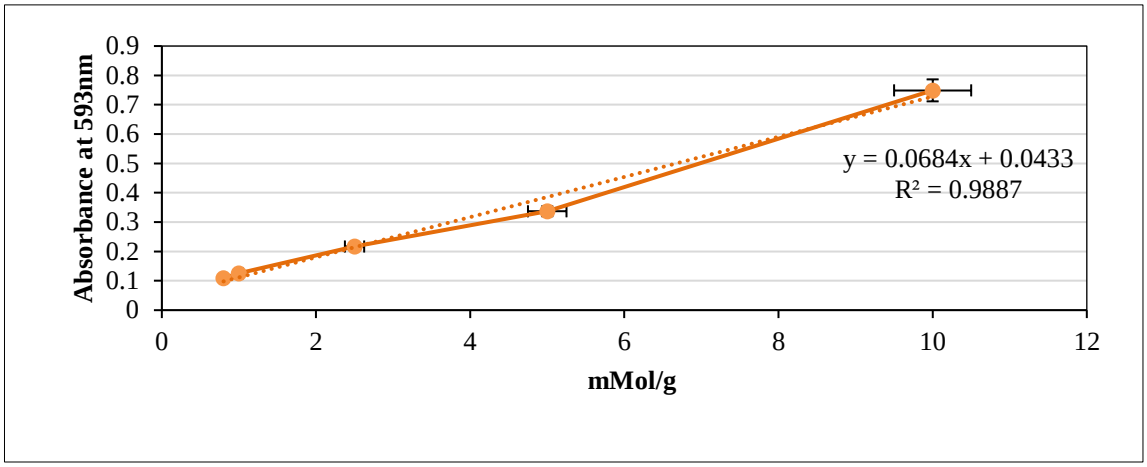


Figure 2.12: Iron sulphate standard curve for FRAP assay.

The results in **Figure 2.12** show $FeSO_{4\times}7H_2O$ was obtained in the range of 0.8 to 10 mMol/g (R^2 = 0.98873). **Table 2.8** shows that at concentrations of (20 mg/ml) the FRAP anti-oxidant activities were highest for DFPE (7.60 mMol/g) compared to ascorbic acid (6.39 mMol/g).

Table 2.8: Ferric Reducing Anti-oxidant Potential Assay (FRAP).

Sample	Concentrations (mg/ml)	Anti-oxidant capacity (mMol/g)
DFPE	20	7.60±0.04
Ascorbic acid	20	6.39±0.11

2.3.7 Anti-nutritional factors analysis

The results for the anti-nutritional factors of (DFPE) are shown in **Table 2.10**

Table 2.9: Percentage of anti-nutritional factors in DFPE and date syrup.

Anti-nutritional factors	mg/100 g dry weight of DFPE powder	mg/100 g wet weight of date syrup
Oxalate	3.90±0.14	4.50±0.11
Tannic acid	0.15±0.07	0.20±0.05
Phytate	0.20± 0.02	0.15± 0.01

The value of oxalate in **Table 2.9** of (3.90 mg/100 g DW) in DFPE is comparable to the value of date fruit (3.6-7.5 mg/100 g DW) reported by Shaba et al., (2015) and Nadeem et al., (2011). Based on 5 individuals on the study reported by Norris (2013) the acceptable daily intake of oxalate is estimated to be ranging from (44-352 mg/day).

The DFPE contains 0.15 mg of tannin 100 g per dry weight which is lower than the values reported for date palm fruit by Shaba et al., (2015) at about (0.53 mg/100 g DW) and higher than the value reported by El-Shemy et al., (2000) for whole seeds of soybean and fababean (0.03 mg/100 g DW). Based on The European Food Safety Authority (2014) a concentration of 15 mg/kg food or feed has been reported to be safe for all animal species.

The phytate content in DFPE was (0.20 g/100 g DW), which is lower than that reported for date palm fruit from Pakistan (0.30-0.77 g/100 g DW) (Nadeem et al., 2011). The content of phytate in DFPE is in the range for whole soybean seeds (0.21 g/100 g DW), whilst it is higher than whole fababean seeds (0.07 g/100 g DW) reported by (El-Shemy et al., 2000). A phytate diet of (10-60 mg/day) has been reported to decrease bioavailability of minerals in monogastric animals (Elinge et al., 2012). This aspect of anti-nutritional factors in an infant cereal product containing DFPE will be discussed further in **Chapter 5**.

2.4 Conclusions

Proximate analysis of the DFPE sample showed that it has a protein content of 25.8%. To the researcher's knowledge, a protein extract from date fruit with such a high level of protein has not been reported before. The concentrations of fat corresponded to levels reported in date fruit before. The concentration of carbohydrate and fibre was less than reported for date fruit, which is explained by the fact that protein was enriched by the extraction, replacing carbohydrate and fibre. The proximate analysis of date syrup showed that it had a protein concentration of 1.6% and carbohydrate concentration of 71.1%.

Amino acid analysis showed that DFPE had all essential and non-essential amino acids, which the body cannot make and must be provided in the diet (Al-Farsi et al., 2005b), although the concentrations of cysteine, methionine and tryptophan was low.

The PDCAAS value of protein in date fruit was reported here for the first time. The PDCAAS for DFPE was 72% and was lower than that of skimmed milk, SPI, casein and egg white. The relatively low PDCAAS value was caused by the low digestibility of DFPE, approximately half of that of SPI. This was attributed to the presence of fibre, oxalate and tannin in DFPE. The PDCAAS of DPFE could be increased by improving the extraction process and increasing the protein purity and concentration.

The mineral analysis of DFPE showed a high iron concentration and lower levels of sodium, calcium, copper, zinc and manganese.

The anti-oxidant activity in DFPE was similar to and even higher than that of ascorbic acid, measured by using the ferric reducing anti-oxidant potential assay (FRAP) and lower than that of ascorbic acid, measured by using radical scavenging assay (DPPH). The high anti-oxidant activity might be attributed to phenolic compounds that were co-extracted from date fruit. Dates contain high quantities of phenolic compounds ranged from 193.7 mg/100 g to 239.5 mg/100 g (Al-Farsi and Lee, 2008a).

Al-Hooti et al., (1997) reported that date palm fruit contains low amounts of protein and is not a good source of protein. In this study a protein extract from date fruit was prepared that could act as a good source of protein, amino acids, minerals and with excellent anti-oxidant properties matching that of ascorbic acid. The levels of anti-nutritional oxalate, phytate and tannin were at low levels and it is assumed that it would not be of concern in infant food. However, the extract had a lower PDCAAS value than SPI which leads to the conclusion that DFPE could not fully replace SPI on a nutritional basis. The PDCAAS of DFPE could be improved by improving the extraction process to provide a purer protein concentrate.

CHAPTER THREE

The effect of heat treatment on the physicochemical and functional properties of date fruit protein extract

3.1 Introduction

3.1.1 Aims of this chapter

This chapter reports the methods and results of the investigation of the effect of heat treatment on DFPE compared to soy protein isolate (SPI) in terms of their functional properties including solubility, foaming, emulsifying ability and water/oil holding capacity. The effect of thermal treatment on physicochemical properties of proteins that underpin their functionality were investigated, which includes determination of free and total sulphydryl groups, surface hydrophobicity and turbidity.

Protein Structure

Protein consists of a chain of amino acids connected via peptide bonds (Berg et al., 2002). The types of amino acid (hydrophobic or charged), their order in the protein chain and how they interact to form a three-dimensional structure in solution, all impact on their functional properties in food systems. A protein's conformation is determined by four structural levels, namely: primary, secondary, tertiary and quaternary.

The primary structure of the protein denotes the linear sequence in which the constituent amino acids are joined via peptide bonds, thus the amino acid sequence of the polypeptide. The secondary structures relate to the pattern of hydrogen bonds between the amino hydrogen and carboxyl oxygen atoms in the peptide backbone. The tertiary structure of a protein is the three-dimensional shape of a protein. The quaternary structure refers to the spatial arrangement of protein containing several polypeptide chains or protein subunits (Damodaran and Paraf, 1997).

3.1.2 Types of proteins

There are two types of proteins in plants, which are distinguished based on their shape: globular or corpuscular proteins such as storage proteins, which are soluble, and the fibrous or fibrillar proteins, such as gluten that are insoluble (Raaman, 2006). They have

distinctly different physicochemical properties. Globular proteins have ovoid or spherical shapes and are generally soluble in water or in aqueous media containing bases, acids, alcohol or salts. Fibrous proteins resemble fibres or long ribbons in shape. These types of proteins are mostly insoluble in aqueous solvents and may be divided into four types: collagen, elastins, keratins, and fibroins (Scheibel, 2005).

In fibrous and globular proteins the peptide chains are bound together by intermolecular hydrogen bonds and in some cases by inter and intra molecular disulphide bonds. Fibrous proteins do not denature as easily as globular proteins. Globular proteins have many metabolic functions and can act as enzymes while fibrous proteins act only as structural proteins. Globular proteins are highly branched or coiled structures and are responsible for the transportation of vital nutrients like oxygen through haemoglobin.

Globular proteins were identified as the main protein type in the DFPE (**Chapter 4, Section 4.4.3)**. The physicochemical and functional properties of globular proteins are described in this section.

3.1.3 Physicochemical properties of proteins

3.1.3.1 Principle of free and total sulphydryl groups

A sulphydryl group consists of sulphur bonded to a hydrogen atom. It also called a thiol, which is an organic complex that includes a sulphydryl group (-SH). It has a great affinity for soft metals. Sulphydryl group plays a significant role in biochemistry, as disulfide bonds connect amino acids jointly for functional purpose in secondary, tertiary, or quaternary protein structures.

Denaturation of globular proteins involves the unfolding of protein molecules during heat treatment, inducing change in the secondary and tertiary structures and exposing the free sulphydryl SH (FSH) groups or thiol groups.

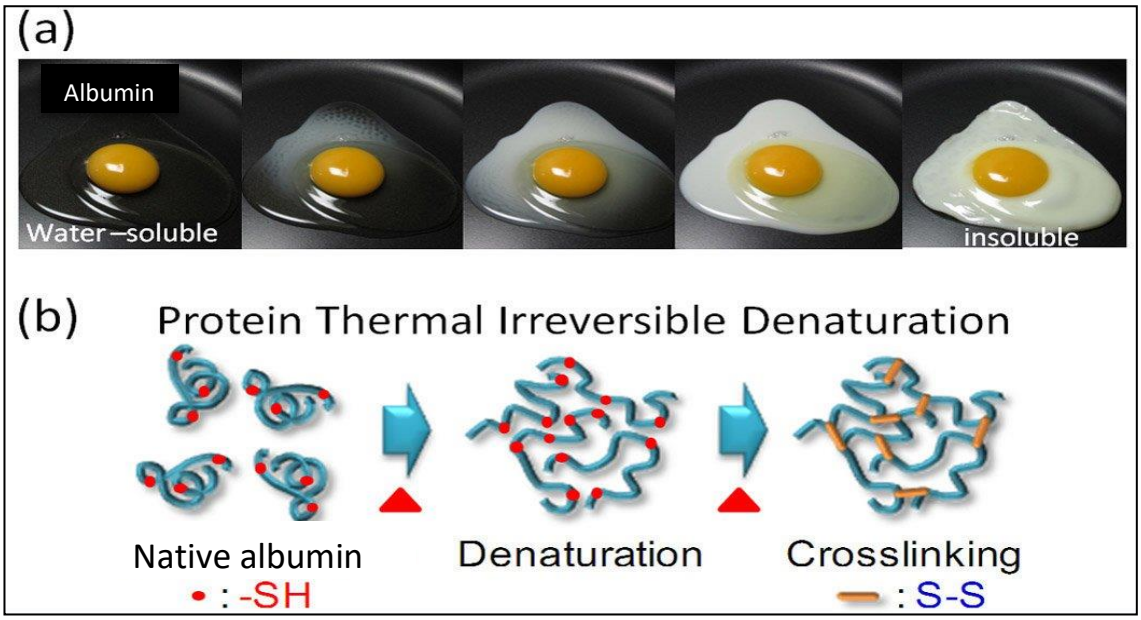


Figure 3.1: The role of thiol groups in denaturation of egg white (Jer-Wei Changet et al., 2012).

During thermal treatment of globular proteins, the free SH groups (FSH) hidden in the interior of the molecule become exposed and as the intensity of the heat treatment increases, they can form inter- and intra-molecular bonds via disulphide bond formation. The extent of disulphide bond formation can only be measured when the protein is completely unfolded. Depending on the intensity of heat treatment, not all the molecules of the protein are completed unfolded and some FSH still remains hidden and is not exposed on the surface. In order to quantify the degree of denaturation, it is essential to determine all the SH groups of the protein, together with those residing within the unfolded structure. Total SH (TSH) is measured by the addition of urea and SDS to complete the unfolding of the protein (Owusu-Apenten, 2005).

Free sulphydryl groups (FSH) is a measure of native and heat-exposed SH groups; it could be a measure of protein denaturation, and thus an indicator of the functional performance of the denatured protein. The FSH and TSH groups are measured by reaction of 5,5'-dithio-*bis*-(2-nitrobenzoic acid), $DTNB^{2-}$ with the sulfhydryl groups of cysteines to form mixed disulfide and TNB^{2-}. The latter compound is yellow and the intensity of the colour

is measured by colorimetry at 420 nm wavelength and quantified as a measure of SH groups (Owusu-Apenten, 2005) see **Figure 3.2**.

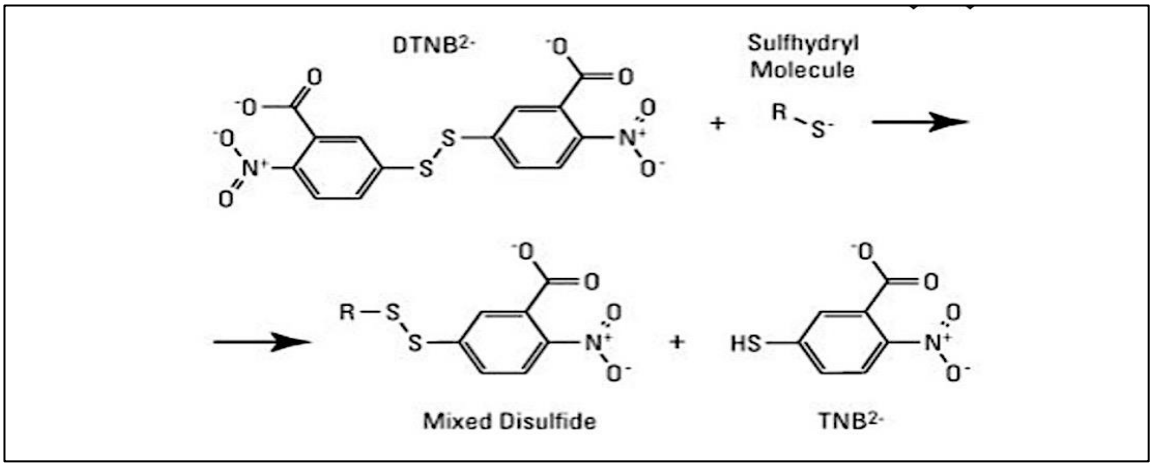


Figure 3.2: The structure for the Ellmann's reagent and its reaction with sulfhydryl groups.

3.1.3.2 Surface hydrophobicity

Hydrophobicity is the molecular driving force behind several necessary biological processes, for instance, protein folding (Li et al., 2007). Hydrophobic interaction is a result of repulsion of water by hydrophobic molecules (Cramp, 2007). Surface hydrophobicity is a structure-related factor affecting the functionality of the proteins (e.g. solubility or gelation properties). Surface hydrophobicity is determined by the solubility of the protein: high hydrophobicity leads to reduced solubility because the hydrophobic amino acid side chains react with each other rather than with water (Wagner et al., 2000). The hydrophobic bonds can be broken by dispersing the protein sample in a solvent containing 8 M urea. According to Zou et al., (1998) urea binds to the amide groups through hydrogen bonds, decreasing the hydrophobic effect through dehydration of the protein molecules.

Principle of method for determination of hydrophobicity

The fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS) is the most common method used to determine the surface hydrophobicity, because of its high sensitivity and simplicity (Al-Amari, 2008). It is used to find out the conformational changes induced by ligand binding in proteins, as ANS's fluorescent properties will change when it binds to hydrophobic regions on the protein surface (Malnasi-Csizmadia et al., 1999).

3.1.4 Functional properties of proteins

Physicochemical characteristics of proteins govern the functional properties of plant proteins, which in turn determine their possible uses in food products (Hermansson, 1979). According to a definition provided by Kinsella and Melachouris (2009), functional properties are "those chemical and physical properties which affect the behaviour of food proteins in food systems during preparation, storage, and consumption". Extrinsic factors can affect the functionality of proteins for example, pH, temperature, ionic strength, and interaction with other components in a solution or food matrix. The most significant functional properties of proteins in food applications are solubility, foaming properties, and emulsifying properties (Yada, 2018).

3.1.4.2 Foaming properties

The property of a protein to make a stable foam is important in producing foods, such as whipped topping products, leavened bakery and meringues (Townsend and Nakai, 1983). Foams are dispersions of gases in liquids and consist of a two-phase system consisting of air cells separated by a thin continuous liquid layer known as the lamellar phase, as illustrated in **Figure 3.3**. Proteins stabilise foams by forming cohesive films around the gas bubbles. The proteins are adsorbed at the interface via hydrophobic areas that are exposed following partial unfolding (surface denaturation). The reduction of surface tension caused by protein adsorption facilitates the formation of new interfaces and small gas bubbles (Ghosh and Bandyopadhyay, 2012).

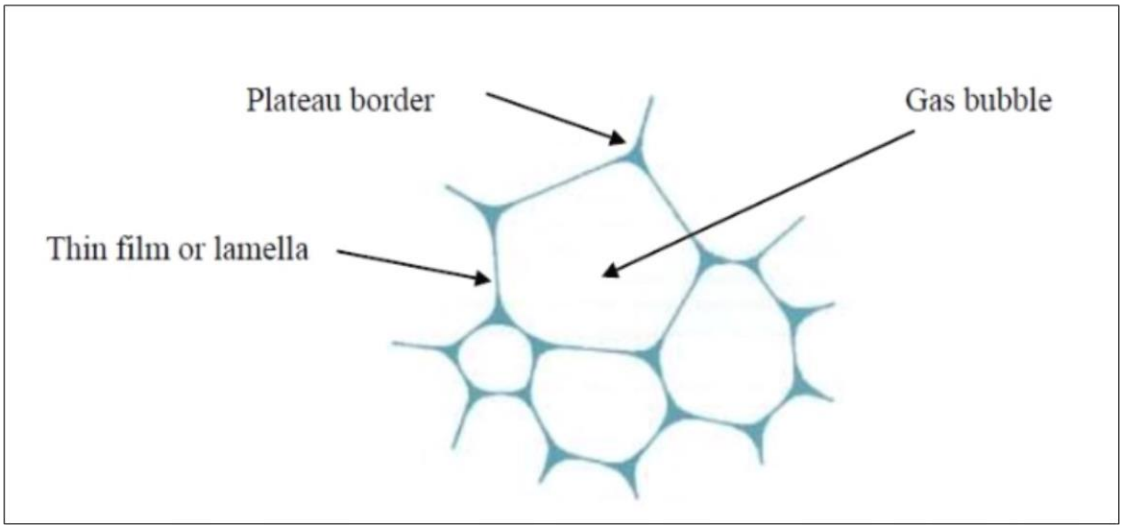


Figure 3.3: The structure of foam bubbles (Wilde and Clark, 1996).

Foaming is characterised by two main parameters, which are foaming stability and foaming ability (Nicorescu et al., 2011). Foam stability is determined by the extent of liquid drainage, which results in decreased foam volume due to bubble coalescence over time. The shelf life of food foams is determined by foam stability. The term foam stability refers to the ability of the foam to retain properties, which are constant over time, such as the liquid content and the volume of foam (Denkov and Marinova, 2006). Foaming ability is the quantity of air phase measured in the protein solution following gas introduction. Foaming ability is evaluated by measurement of the increase in the volume of foam when whipping (Ibanoglu and Ercelebi, 2007).

3.1.4.1 Solubility

The solubility of the protein is the primary functional property and is defined as the ability to associate with water. Solubility can also be defined as the quantity of the protein remaining in the supernatant after the dispersion has been centrifuged for a period of time (Pelegrine and Gasparetto, 2005). Protein solubility is a basic requirement for its performance in several food products, including foaming, gelation and emulsification properties (Zayas, 1997). The solubility of the protein is influenced by environmental factors, such as pH, solvent type, temperature, processing conditions and the ionic strength of the solution (Kasran et al., 2013; Ahmed, 2013). It is a function of the amino

acid composition and conformation of proteins, including hydrophobic and hydrophilic properties (Zayas, 1997).

Effect of solvent type

The number of polar and non-polar groups and their arrangement along the molecule affects protein solubility. In general, globular protein is very soluble in strong polar solvents (e.g. water, formamide, glycerol, formic acid or dimethylformamide), while it is rarely soluble in less polar solvents (Pace et al., 2004). The solubility of the proteins in polar solvents is dependent on pH and salt concentration (Belitz et al., 2009).

Effect of ionic strength of protein solution

The solubility of a protein rises with increase in ionic strength, due to the preferential binding of anions to the protein (Belitz et al., 2009). At low concentrations of salts, such as sodium sulphate or sodium chloride, the solubility is increased due to the suppression of the electrostatic protein-protein interaction, while it is decreased at higher salt concentrations (above 1.0 M) because of the ion hydration tendency of the salts (Zayas, 1997; Nahar et al., 2017).

Effect of pH of protein solution

The level of solubility in the aqueous medium is the result of electrostatic and hydrophobic interactions between the protein molecules. The solubility of proteins is influenced by a sensitive balance between repulsive and attractive intermolecular forces; protein is soluble when electrostatic repulsion between proteins is higher than hydrophobic interactions (Zayas, 1997). The concentration of soluble protein increases as pH increases and it decreases as pH decreases. The iso-electric point (pI) of the protein is the pH at which its net electrical charge is zero, at pH below the pI proteins will carry a net positive charge while above the pI proteins will carry a net negative charge (Bhatia et al., 2015). Thus, the solubility of proteins depends on the net charge on the surface of the proteins. A protein becomes more soluble if there is a net charge at the protein surface,

due to its preference to interact with water rather than with other protein molecules. "Phenylalanine, tyrosine and tryptophan are hydrophobic by virtue of their aromatic rings while the amino group on the side-chains of arginine and lysine are protonated and thus positively charged at neutral pH. The side-chain of histidine can be positively charged or uncharged at neutral pH. In contrast, at neutral pH the carboxyl groups on the side-chains of the acidic amino acids aspartic acid and glutamic acid are de-protonated and possess a negative charge" see **Figure 3.4** (Hames and Hooper, 2011).

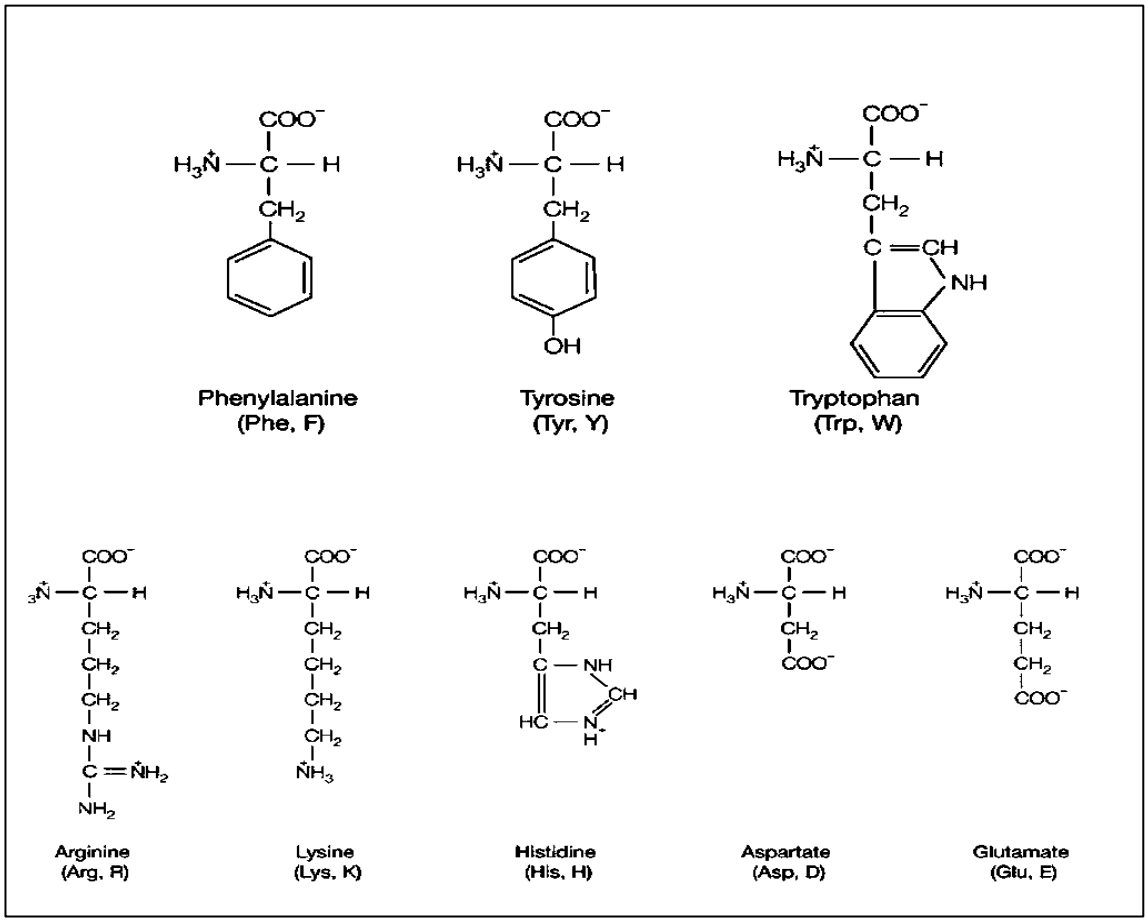


Figure 3.4: The hydrophobic/aromatic amino acids and the charged amino acids are illustrated in the upper and lower parts of the figure, respectively (Hames and Hooper, 2011).

Effect of thermal treatment on protein solubility

Protein solubility is determined by the degree of the denaturation during heat treatment, which in turn determines its functionality in food systems (Kinsella, 1967).

The solubility of globular proteins is reduced with increased denaturation during thermal treatment and can lead to precipitation. Mu et al., (2011) reported that exposure of some native globular proteins to extreme temperatures leads to unfolding of the quaternary and tertiary structures. The hydrophobic groups that are initially buried in the interior of the protein structure are exposed, leading to aggregation and precipitation.

The solubility of some globular proteins at protein concentration lower than 10% is increased by exposure to a temperature between 0°C and 40-50°C, while it is decreased at temperatures higher than 40-50°C (Zayas, 1997). The degree of the protein's solubility reflects the extent of denaturation and aggregation of protein, which can affect the foaming, gelling and emulsification properties of protein.

3.1.4.4 Emulsifying properties

Emulsifying properties of proteins are their ability to stabilise an oil in water emulsion and are necessary for various food applications, such as cakes and mayonnaise (Jackman et al., 1989). According to Belitz et al., (2009): "Emulsions are dispersed systems of one or more immiscible liquids. They are stabilised by emulsifiers that form interfacial films and thus prevent the dispersed phase from flowing together". An emulsion is created from one incompatible phase distributed as little droplets within the matrix of a second phase by means of an emulsifying agent. There are two basic types of emulsions, which are water in oil (W/O) emulsion and oil in water (O/W) emulsion. The (O/W) emulsions normally exhibit a creamy texture, whereas a (W/O) has a greasy texture (Zayas, 1997). In addition to these two types, there are some emulsions that consist of many phases that can be prepared by dispersing oil-in-water or water-in-oil emulsions in another liquid

medium, which may be water or oil: these types of emulsions are called multiple emulsions (Khan et al., 2011).

Globular soluble proteins act as emulsifiers by decreasing the interfacial tension between hydrophilic and hydrophobic components, leading to reduced size of the oil droplets in emulsion, which leads to an increase in the viscosity and emulsion stability (Fletcher, 2015). The emulsifying capability (EC) is the ability of the protein solution to emulsify oil, which depends on its ability to form adsorbed films around the fat globules. Emulsifying stability (ES) is the capability of emulsion droplets to stay stable while not separating due to coalescence, flocculation or creaming (Homoud, 2015). See **Figure 3.5**.

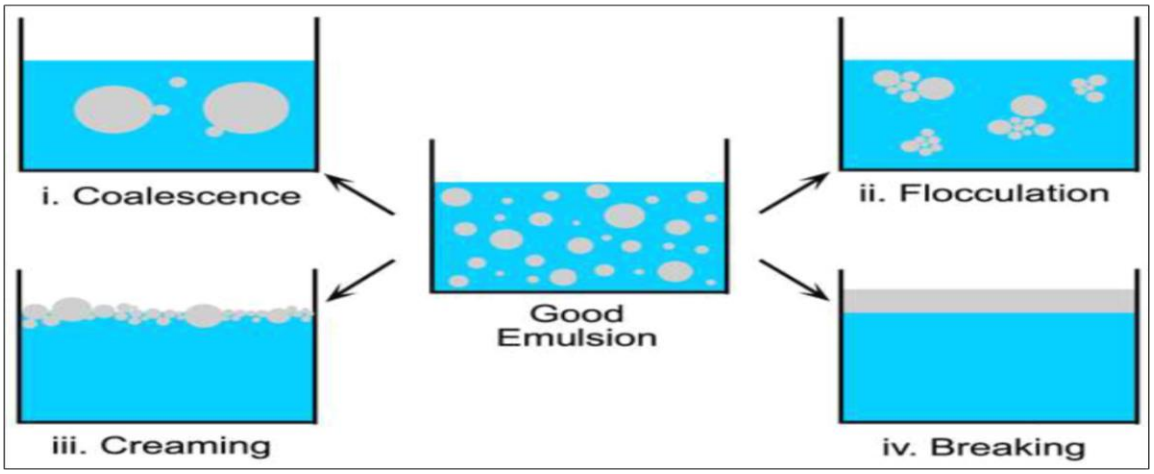


Figure 3.5: Instability types of emulsion (Zayas, 1997).

3.1.4.5 Water absorption capacity (WAC)

Huang and Kinsella (1986) used the term of water absorption capability (WHC) to refer to a quantitative indication of the quantity of water maintained by a protein in solution under defined conditions Kneifel et al., (1991). WAC of a protein is its ability to absorb and store water, which is a significant component of many foods, e.g. meat products and dough (Kinsella and Melachouris, 2009). It can be determined by centrifugation of the protein matrix and measurement of the separated water (Zayas, 1997). Water absorption capability depends on many factors, e.g. the carbohydrate fractions associated with the protein and the hydrophilic–hydrophobic balance of amino acids in the protein molecule

(Chou and Morr, 1979). The WAC of proteins in solution differs from that water absorption capacity (WAC) of a protein powder (see **Section 3.1.4.7**).

3.1.4.6 Oil absorption capacity (OAC)

Oil absorption capacity (OAC) is a necessary function for some applications, such as meat replacers and extenders, mainly because it enhances flavour and improves mouth-feel. Oil absorption capacity is measured by adding liquid oil to a protein solution and blending it very well then centrifuging it for 30 min to discard the excess of oil and finally determining the amount of bound oil. This is expressed on a per 100 g protein basis (Kinsella and Melachouris, 2009). The oil absorption capacity of protein depends on the content and the type of hydrophobic areas in the protein, as well as the presence of hydrophobic amino acids in the structure of protein. The presence of many non-polar side chains can bind the hydrocarbon chains of oil, leading to higher oil-binding capacity (Thanatcha and Pranee, 2011).

3.1.4.7 The water and oil absorption capacity (WAC and OAC) of food protein powders

The functional properties of powders are linked to the interaction between water/oil and powder. They are influenced by protein structure, protein surface and the interaction with other food components in the dry powder. These functional properties are dependent on the specific surface area, which is related to structural parameters the total pore volume, porosity and particle size (Nguyen et al., 2015). The two main functional properties determined to assess the functionality of protein powders are water and oil absorption capacity. High water absorption of proteins helps to maintain freshness and moist mouth feel of baked goods and are considered critical in viscous foods, such as soups. High oil absorption is essential in food systems like sausages and cake batters (Chandi and Sogi, 2007).

3.2 Chemicals and methods

Chemicals

Table 3.1: Chemicals obtained from Sigma Aldrich, UK

Chemicals obtained from Sigma Aldrich, UK	Catalogue number
K-phosphate buffered saline pH 7.5	P4417
NaOH	1310-73-2
HCl	7647-01-0
Bovine serum albumin (BSA)	9048-46-8
Bradford reagent	B6916
Sodium dodecyl sulphate ($NaC_{12}H_{25}SO_4$) (SDS)	151-21-3
Urea (CH_4N_2O)	57-13-6
Ethylenediaminetetra-acetic acid (Na_2EDTA)	60-00-4
Glycine ($C_2H_5NO_2$)	56-40-6
Tris Base ($C_4H_{11}NO_3$)	77-86-1
5,5′-dithio-*bis*-2- nitrobenzoic acid ($C_{14}H_8N_2O_8S_2$) ($DTNB^{2-}$)	69-78-3
8-anilinonaphthalene-1-sulphonic acid ($C_{16}H_{13}NO_3S$) (ANS)	82-76-8

Methods

The description of all the methods used in this study for determining physicochemical and functional properties, is presented as follows.

3.2.1 Physicochemical properties

3.2.1.1 Free and total sulphydryl groups

The sulphydryl (SH) content of (1.25%) protein solutions of DFPE and SPI, (non-heated samples RT and heated samples at 80°C for 15 at 30, 45 and 60 min) was determined by a colorimetric assay using 5,5′-dithio-*bis*- 2-nitrobenzoic acid ($DTNB^{2-}$), as described by Campbell et al., (2009).

For the determination of the concentration of the thiol groups, 300µl of the sample was added to 2 ml Tris-glycine buffer (0.086 M Tris, 0.09 M glycine, 0.02 M Na_2EDTA, pH 8), followed by the addition of 200 µl 0.02 M DTNB buffer. The solution was vortexed and left to react at RT for 15 min before recording the absorbance at 412 nm, using a spectrophotometer (Model Genesys 6, Thermo Electron Corporation, USA). The blank

for each measurement was the date extract or SPI solutions' sample prepared using the described procedure but omitting the DTNB.

For determination of the concentration of total thiol groups, 300 µl of each sample was added to 2 ml Tris-glycine buffer pH 8 containing 6 M urea and 0.5% SDS to fully denature the protein. 200 µl of 0.02 M DTNB buffer was then added and absorbance at 412 nm was measured as described for free SH-groups.

The calculation of molar quantity of SH groups in the protein was calculated according to the following equation:

Concentration of SH groups (µMol/g protein) = $(A * Vt)$ / (13700 * Vs) * 1/0.004

where A is absorbance of the sample, Vt is the total volume of the sample in the cuvette, Vs is the volume of date extract within the sample in the cuvette, 1/0.004 is 1 divided by the protein concentration (gram) of the sample in Vs and 13700 is ε = ($M^{-1}cm^{-1}$) of the 5,5'-dithio-*bis*-(2-nitrobenzoic acid)

3.2.1.2 Surface hydrophobicity

The hydrophobicities of DPFE and SPI were determined using ANS (8-anilino-1-naphthalene sulphonic acid) as the fluorescence probe, according to Shih et al., (2015), with slight modifications. Aliquots of a protein solution (1.13%) were heat-treated at 80°C for 15 and 45 min. 200, 300, 400 and 500 µl of DFPE and SPI solutions were made up to 2 ml using 0.01 M phosphate buffer at pH 7.0. 15 µl of ANS (8.0 mM in 0.1 M phosphate buffer, pH 7) was then added and the solutions left to stand for 1 h at room temperature. Fluorescence intensity (FI) was measured using a spectrophotometer (Model 203, Stable Micro System Ltd.) at wavelengths of 390 nm excitation and 470 nm emission. The non-heat-treated samples for both date and soy extracts were used as the control. The initial slope of the plot of fluorescence intensity versus protein concentration was used as an index of hydrophobicity.

3.2.1.3 Turbidity

Turbidity was determined according to the method described by Tay et al., (2005), with the modification of intensity of heat treatment. Whereas the published heat treatment was 100°C for 10 min, heat treatment in this experiment consisted of heating 100 ml of 1.13% protein solutions (DFPE and SPI) at 80°C for 15, 30, 45 and 60 min followed by cooling on ice. A non-heated sample was used as a control. The absorbance of 1 ml of the supernatants of the centrifuged samples was measured at wavelength of 600 nm using a Genesys 6 spectrophotometer (Thermospectronic, USA).

3.2.2 Functional properties

3.2.2.1 Solubility

For determining the effects of pH and temperature on DFPE solubility, each protein sample, 2 g containing (5.5%) protein, was suspended in 200 ml of dH_2O (final concentration of 2.5%). It was divided into 2 x 100 ml samples and the pH was adjusted using 0.1 M HCl or 0.1 M NaOH to a pH of 6 and 7 respectively, using a pH meter (Handheld Water-Resistant pH Meter, Romania). The mixtures were then stirred using a magnetic stirrer for 30 min at room temperature. Each 100 ml batch was divided into 20 ml aliquots, which were each pipetted into glass test tubes. One of the 20 ml samples of each 100 ml batch was not heat treated and the other 4 samples were heated at 55, 65, 75 and 85°C, respectively, in a shaking water bath (GLS Aqua18 Plus, Grant instruments Ltd, UK) for 10 min, then cooled on ice, followed by centrifugation at 6000 rpm for 20 min at RT. The soluble protein contents were determined by the Bradford assay, as described by Bradford (1976) (see **Chapter 4, Section 4.3.4**) and the percentage of protein solubility was then calculated as follows:

$$Solubility\ (\%) = \frac{\text{Protein content of the supernatant (mg/ml)}}{\text{Total protein content of the sample (mg/ml)}} * 100$$

3.2.2.2 Foam capacity and foam stability (FC and FS)

Foaming capacity (FC) and foam stability (FS) were determined by the method described by Shaba et al., (2015), with some modifications. 100 ml of a 1.13% protein solution that was not heated or heat-treated DFPE (80°C for 15, 30, 45 and 60 min) were whipped for 5 min and the volume was recorded before and immediately after foaming. Soy protein solution (1.13% protein) was used as positive control.

FC was expressed as percentage using the following formula:

$$FC = \frac{\text{Volume after whipping} - \text{Volume before whipping}}{\text{Volume before whipping}} * 100$$

The foam stability of the sample was recorded by measuring the height after 30 min of whipping and was expressed as a percentage using the following formula:

$$FS = \frac{\text{Foam volume after 30 min}}{\text{Initial foam volume}} * 100$$

3.2.2.3 Emulsion activity index and emulsion stability index (EAI and ESI)

The emulsions were prepared to determine the emulsifying activity (EAI) and the emulsifying stability (ESI) of DFPE and SPI (as control), based on methods described by Kinsella (1979), Liu et al., (2008) and Ogunwolu et al., (2009), with modification of heat treatment of the samples before testing.

50 ml of 1.13% protein solution was heat-treated at 80°C for (15, 30, 45 and 60 min) and 50 ml of water was used as a negative control. Vegetable oil (30 ml) was added to the solutions then the solutions were homogenized at 13000 rpm for 2 min using (IKA 3720001 T-18 Ultra Turrax Digital Homogenizer). 100 µl of the emulsions were added immediately and 10 min after homogenisation to 3 ml of 0.1 % SDS solution. The absorbance of each diluted sample was measured at 500 nm using a spectrophotometer (Model Genesys 6, Thermo Electron Corporation, USA).

The EAI and the ESI were calculated using the following formula, according to Pearce and Kinsella (1978):

$EAI\ (m^2/g) = 2 \times T / \emptyset \times C$

$ESI\ (min) = A_0/A_0 - A_{10} \times \Delta t$

where, T = 2.303,), Ø = the oil volumetric fraction (0.25), C = the weight of protein per unit volume (g/ml), A_0 and A_{10} = the absorbance after 0 and 10 min of homogenisation, Δt = 10 min.

3.2.2.4 Stability of oil-in-water (O/W) emulsions

Oil-in-water (O/W) emulsions of DFPE and SPI (as positive control) were prepared by heating 50 ml of 1.13% protein solutions at 80°C for 15, 30, 45 and 60 min, using 50 ml of water as negative control. The solutions were homogenized as above, and the samples were added in duplicate to calibrated 100 ml bottles to measure the water separation visually. After 24 hours of incubation at room temperature, the water phase released from the emulsion was measured by the following formula:

$$WP = \frac{\text{The volume of water at the bottom of the emulsion after the incubation for 24 h (ml)}}{\text{Volume of the whole sample before incubation}} * 100$$

3.2.2.5 Water absorption capacity (WAC)

Water absorption capacity was determined according to the method described by AOAC (2006), with some modification. Samples of 1 g (25.8% protein) of freeze-dried DFPE as well as 0.258 g of SPI were weighed into pre-weighed 15 ml centrifuge tubes, mixed with 10 ml of dH_2O and vortex mixed for 2 min (final protein concentration 2.58%). The samples were allowed to stand at RT for 30 min and then centrifuged at 10000 rpm for 20 min at room temperature. The supernatants were gently discarded, and the pellets were weighed.

The water absorption capacity was calculated by following the formula:

$$WAC = \frac{\text{Weight of sample after centrifugation (g)} - \text{Weight of empty centrifuge tube (g)}}{\text{Weight of sample before centrifugation (g)}} * 100$$

3.2.2.6 Oil absorption capacity (OAC)

Oil absorption retention was determined according the method described by AOAC (2006) with some modification. Samples of 1 g (25.8% protein) of freeze dried DFPE and 0.258 g SPI powder were weighed into pre-weighed 15 ml centrifuge tubes and mixed with 10 ml of sunflower oil and vortex mixed for 2 min (final protein concentration 2.5%). The samples were allowed to stand at RT for 30 min and then centrifuged at 10000 rpm for 20 min at RT. The supernatants were gently discarded, and the pellets were weighed and recorded.

The oil absorption capacity was calculated by the following formula:

$$OAC = \frac{\text{Weight of sample after centrifugation (g)} - \text{Weight of empty centrifuge tube (g)}}{\text{Weight of sample before centrifugation (g)}} * 100$$

3.3 Results and discussion

3.3.1 Comparison of protein concentrations used for different functionality tests

Table 3.2: Protein concentrations used for functionality tests

Functionality test	Protein concentration %
Solubility	2.50
Water absorption capacity and oil absorption capacity	2.58
Foaming capacity and foaming stability, turbidity	1.13
Emulsion activity index and emulsion stability index	1.13
Stability of oil-in-water (O/W) emulsions	1.13
Turbidity and hydrophobicity	1.13
SH groups	1.25

3.3.2 Physicochemical properties

3.3.2.1 Effect of heat treatment on free and total sulphydryl groups

Free sulphydryl (FSH) groups of proteins are measured by a colour reaction in buffered solution. The number of FSH groups of globular proteins, such as SPI and whey protein increases when heat-treated, due to the unfolding of the protein. Total sulphydryl (TSH) groups are measured in a solution containing denaturing agents that fully denature protein without breaking the disulphide bonds. Measurement with colour reagent gives an indication of TSH groups in the unfolded protein. Extensive thermal treatment would result in reduction of TSH groups due to formation of disulphide bonds.

The results presented in **Figures 3.6** and **3.7** show that the free and total SH of DFPE for the non-heated samples was 2.22 and 3.09 μMol/g protein, respectively. This is the first time that the concentration of free and total SH groups of date fruit protein has been reported. In comparison the free and total SH of SPI for the non-heated sample was 3.43 and 4.70 μMol/g protein, respectively. These values are in a similar range than those reported by Campbell et al., (2009) of 5.1 and 14.8 μMol/g protein, respectively for SPI. The values for both free and total SH of DFPE were 35% lower than those for SPI. This corresponds to lower contents of cysteine in date protein than those for SPI, as reported by Al-Farsi and Lee (2008a) **Chapter 2, Table 2.2 and Figure 2.8** also reports a relatively low value for cysteine 25 mg/100g.

The results in **Figure 3.6** in this chapter indicate that heat treatment at 80°C for up to 60 min did not affect the concentration of FSH for DFPE. On the other hand, SPI showed a significant increase ($p < 0.05$), from 3.3 to 5.24 μMol/g protein, supporting the findings of Gu et al., (2009) who demonstrated an increase in FSH after thermal treatment of 8% SPI, which could be attributed to the increased availability of surface SH-groups as the protein unfold.

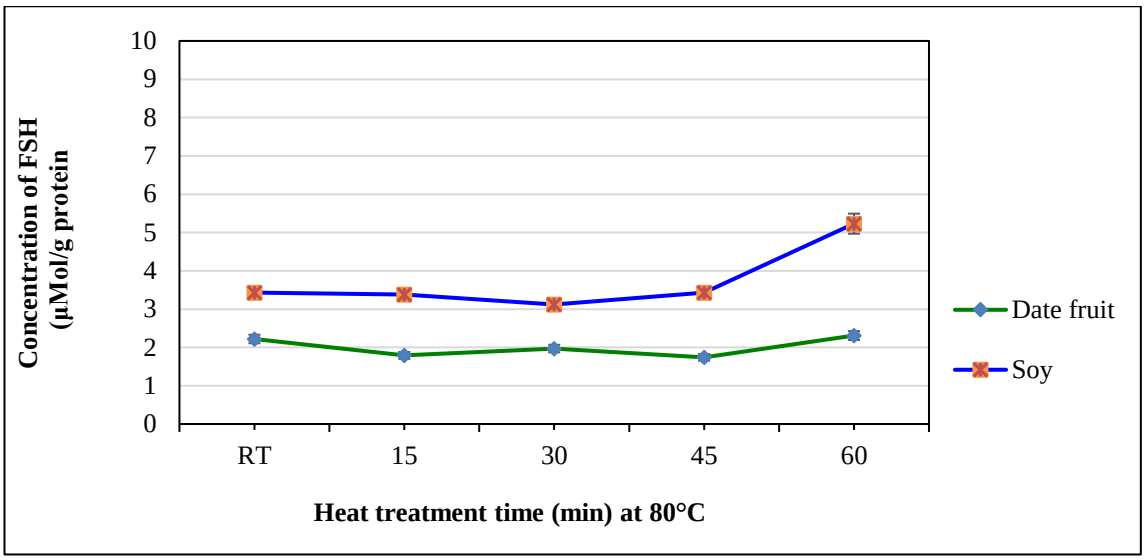


Figure 3.6: Effect of heat treatment on free sulfhydryl groups of DFPE compared to SPI, 1.25% protein, at pH 7.0.

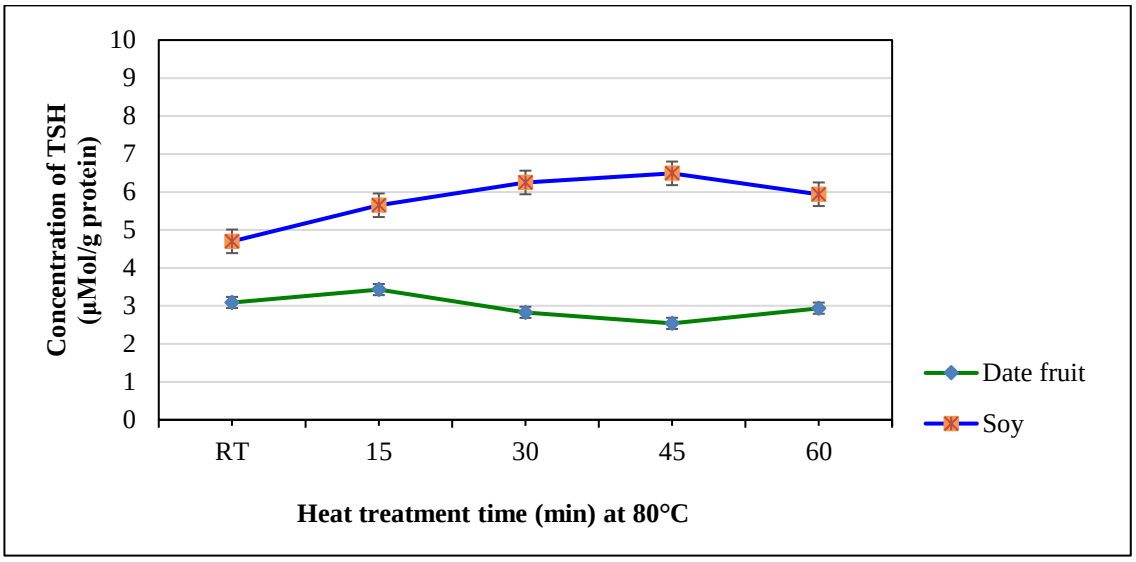


Figure 3.7: Effect of heat treatment on total sulphydryl groups of DFPE compared to SPI, (1.25% protein) at pH 7.0.

Figure 3.7 shows a significant decrease ($p < 0.05$) in TSH for DFPE after 30 and 45 min of heat treatment at 80°C compared to the non-heated sample from (3.09 at RT to 2.83 µMol/g protein after min 30 min and 2.54 µMol/g protein after 45 min heat treatment at 80°C). This could be due to formation of disulphide bonds as a result of denaturation. The increase in TSH after 60 min is within the scope of experimental variation as it is not significant compared to the value for 45 min and is still lower than the result for the control. These results indicate that DFPE heat stability is limited to 15 min heat treatment

at 80°C at a concentration of 1.25% before the functionality would be affected. The susceptibility to heat treatment could increase as the protein concentration increase. Nevertheless, the thermal treatment conditions in the range of 15 min at 80°C would be sufficient to pasteurise food products containing DFPE.

In comparison, the TSH for SPI increased significantly after 15 min. This could be due to urea not fully denaturing the protein when added at RT, which increases in the presence of thermal treatment. Nevertheless, no decrease in TSH was observed for SPI as compared to DFPE, indicating that no disulphide bonds formed at SPI was heated at a protein concentration of 1.25%. This confirms the results of Gu et al., (2009) who showed no decrease in TSH for thermally treated 8% SPI in a similar temperature range. These results indicate that SPI is more stable to thermal treatment than DFPE. However, it has to be taken into consideration that DFPE has been subjected to thermal treatment during the extraction process, whereas SPI has not.

3.3.2.2 Effect of heat treatment on surface hydrophobicity

Hydrophobicity is one of the most significant factors affecting the functional properties of proteins and it increases as globular protein denatures (Hua et al., 2005). **Figure 3.8** shows that the hydrophobicity of SPI increased as a function of heating time at 80°C. On the other hand, heat treatment did not have any effect on the hydrophobicity of DFPE and the values were steady in both heated and non-heated samples. This non-responsiveness of DFPE hydrophobicity could be due to the presence of carbohydrates, including sugars, which are present in DFPE, which could protect the protein from exposing hydrophobic groups. Gu et al., (2009) reported that thermal treatment of 8% SPI solution in the presence of sugars (sucrose, lactose, glucose) significantly reduced its hydrophobicity.

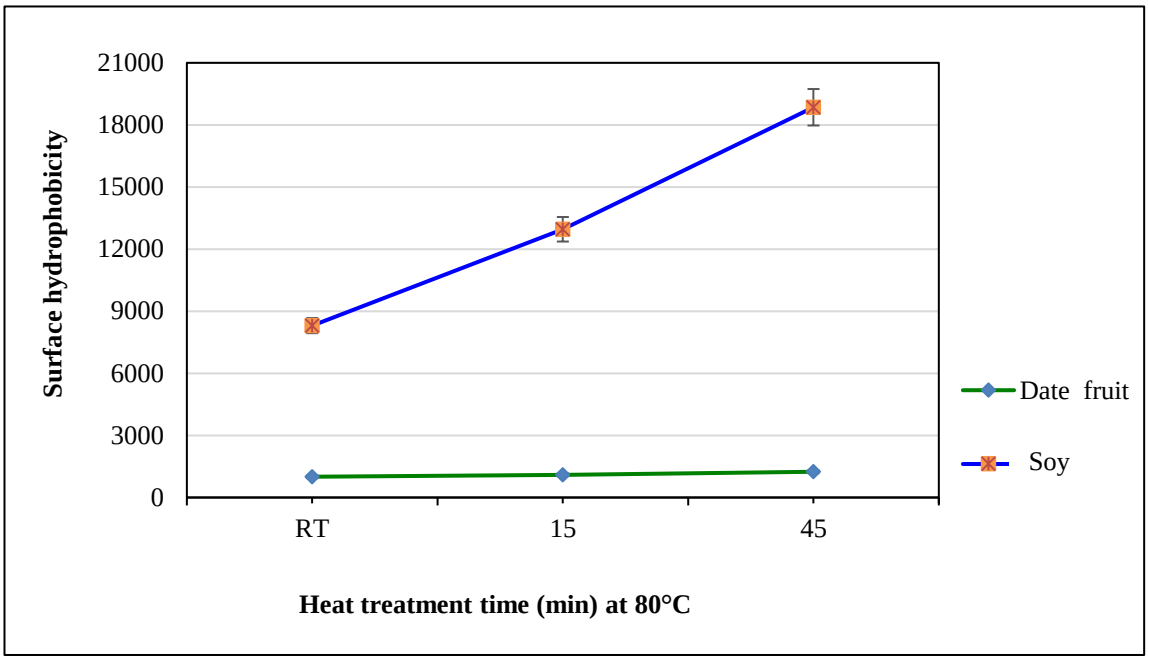


Figure 3.8: Effect of heat treatment on surface hydrophobicity of DFPE compared to SPI, (1.13% protein) at pH 7.0.

3.3.2.3 Effect of heat treatment on turbidity

The results in **Figure 3.9** indicate that samples at RT had a higher turbidity value than the heated samples for DFPE.

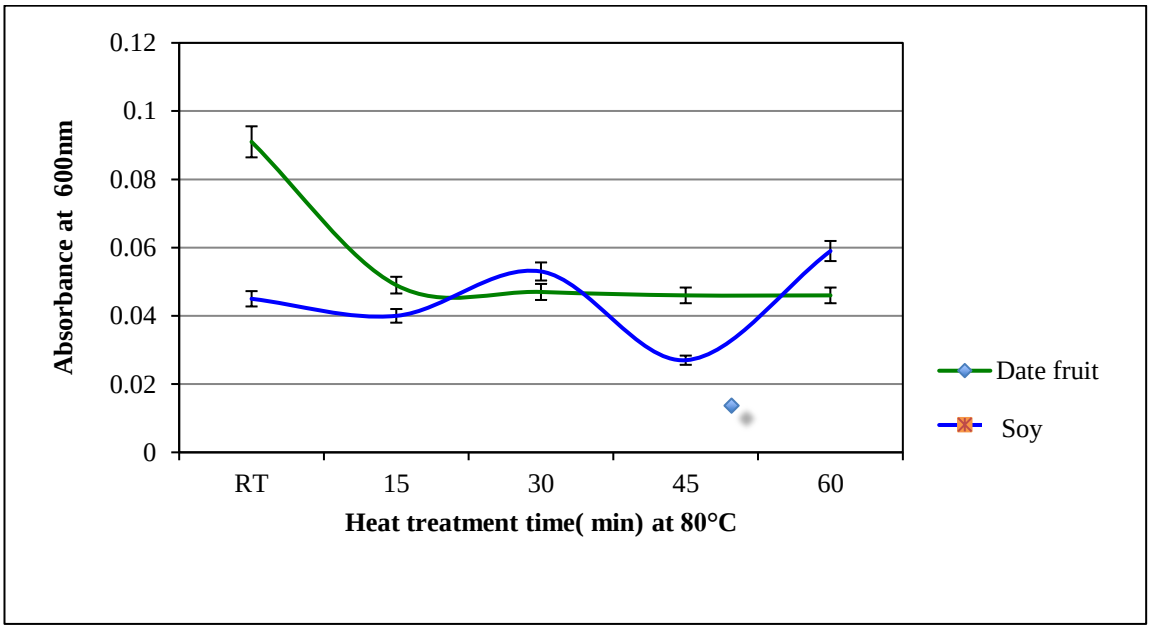


Figure 3.9: Effect of heat treatment on turbidity of DFPE compared to SPI (1.13% protein) at pH 7.0.

Increased turbidity measurements of protein that has been thermally treated would indicate the formation of soluble protein aggregates. The results in **Figure 3.9** indicate that DFPE did not form aggregates, as the turbidity decreased after 15 min heat treatment and remained constant. The decrease in turbidity could be due to solubilisation of carbohydrates and sugars in the solution. SPI, on the other hand, shows a significant increase ($P>0.05$) in turbidity from (0.045) at RT to (0.053) after 30 min heating at 80°C and thereafter, which could be due to aggregate formation **(Figure 3.9)**.

These results confirm the differences in physicochemical properties of DFPE compared to SPI. One of the main reasons for the differences could be the relative impurity of DFPE, which contains 50% carbohydrates, including sugars (**Chapter 2, Figure 2.6**) compared to SPI (5% lactose). Baier and McClements (2001 and 2005) postulated that "the presence of solutes, such as sugars in the aqueous phase of food systems can alter the conformation and interactions of proteins by binding to protein surface groups, or they may indirectly influence these characteristics by altering the physicochemical properties of water. The interactions and their influence on protein functionality depend on the type and concentrations of solutes present".

3.3.3 Functional properties

3.3.3.1 Effect of pH and heat treatment on protein solubility

Protein solubility determines the foaming, gelling and emulsifying ability and also the water binding properties in foods (Mu et al., 2011). **Figure 3.10** presents the experimental data on the effect of pH (6 and 7) and heat treatment on the protein solubility of DFPE compared to that of SPI.

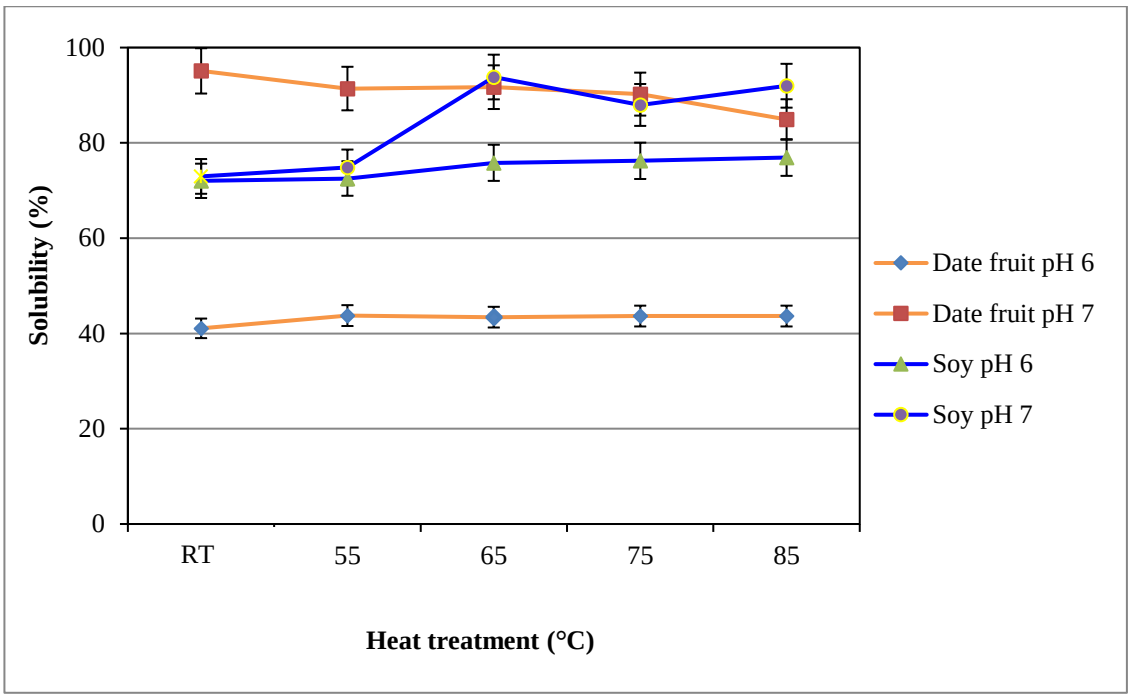


Figure 3.1: Effect of heat treatment at pH 6.0 and 7.0 on protein solubility (2.5% protein).

The graphs show that DFPE was significantly more soluble at pH 7 than pH 6. The highest solubility for DFPE was 95.11% at pH 7, at RT, which was 60% higher than at pH 6. DFPE solubility decreased significantly at pH 6, reason being that it is close to it iso-electric (pI). Al-Khayri et al., (2017) demonstrated that the pI of protein extracted form date fruit lies in the region of between 4.8 and 5.8. This is higher than the pI of soy protein, which is at 4.6 (Liu et al., 2015), which would explain why the solubility of soy protein at pH was not reduced to the same extent. The closer the pH of a protein is to its pI, the more insoluble it becomes. These results indicate that the solubility of DFPE will be reduced at pH below 6 compared to SPI; therefore, it will not be suitable for applications in acidic products such as fruit juice or mayonnaise.

DPFE solubility remained resistant to heat treatment at both pH. The pH 7 sample only showed a significant decrease ($p < 0.05$) at 85°C (84.91%) compared to the control (95.11%), confirming the results of total SH (**Figure 3.7**) remaining stable for 15 min heat treatment at 80°C. The results confirm that DFPE could withstand pasteurisation temperatures at pH 6 and 7.

In comparison, the solubility of SPI at pH 6 and 7 at RT was lower than that of DFPE but increased above 65°C at pH 7. This could be explained because SPI is more susceptible to denaturation than DFPE at temperatures above 65°C, as shown by the increase in turbidity (**Figure 3.9)**. The higher solubility of DFPE compared to SPI at pH 7 could also be attributed to the presence of sugars and carbohydrates in DFPE.

3.3.3.2 Effect of heat treatment on foaming capacity and foam stability

The results of the effect of heat treatment on the foaming capacity and stability of DFPC and SPI are set out in **Figures 3.11** and **3.12**, respectively.

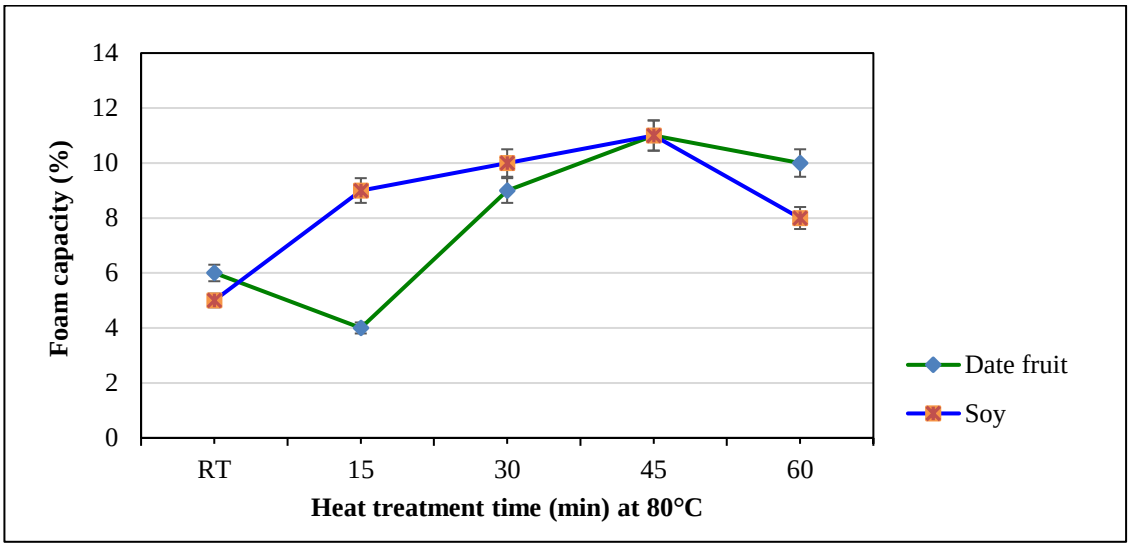


Figure 3.2: Effect of heat treatment on foaming capacity of DFPE compared to SPI (1.13% protein) at pH 7.0.

The foam capacity of DFPE increased significantly ($p < 0.05$) compared to the control (6%) after 30, 45 and 60 min of heating at 80°C, reaching a similar level to SPI at 45 min heating time (**Figure 3.11**). In comparison, the FC of soy protein showed a decreased after 60 min heating. This pattern is comparable to FC of egg white proteins, which increased at 60°C but decreased after heating above 80°C (Gharbi et al., 2017).

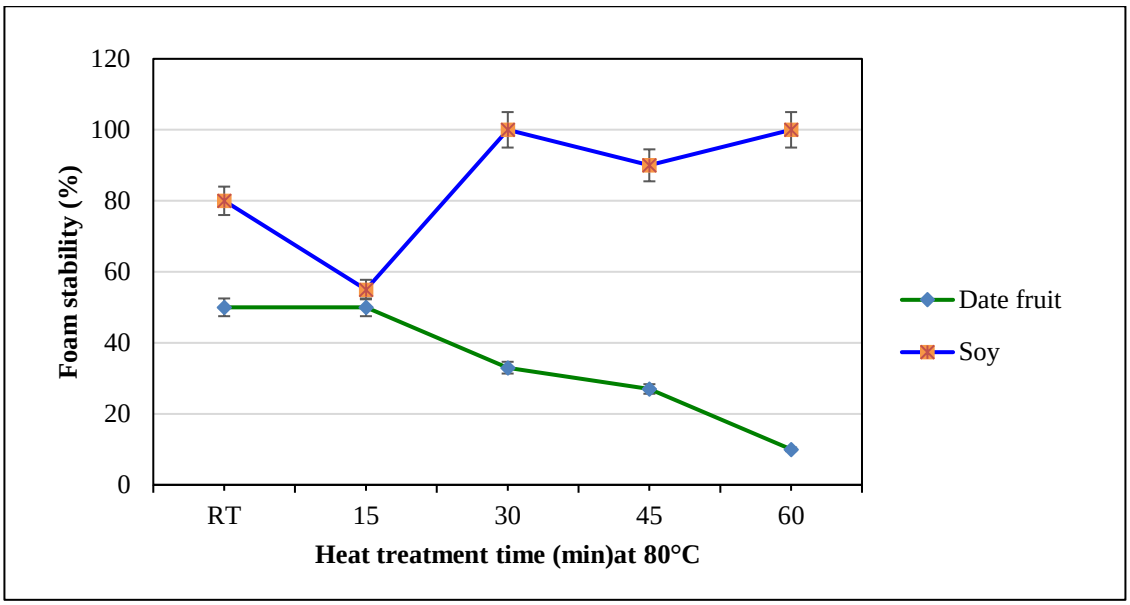


Figure 3.3: Effect of heat treatment on foam stability of DFPE compared to SPI (1.13% protein) at pH 7.0.

The foam stability for DFPE was significantly reduced with increased heating time (**Figure 3.12**). This could be due to the formation of intra-molecular disulphide bonds after 15 min heating time at 80°C (**Figure 3.7**), reducing its ability to unfold at the air/water interface. It could also be due to the competition between proteins and low molecular-weight peptides as well as for non-protein, such as phospholipids for adsorption at the air-water interface leading to producing interfacial films with poor foam stability (Garofalakis and Murray 2001). SPI had comparatively better foam stability than DFPE. This could be because SPI was a pure protein (90%) and had fewer contaminants to destabilise the emulsion. The positive effect of thermal treatment on the foam stability for SPI correlates to increased FSH (**Figure 3.6**) hydrophobicity (**Figure 3.8)** and solubility **(Figure 3.10),** which indicate denaturation of SPI.

These results indicate that DFPE has similar foaming capacity and foam stability than SPI after thermal treatment of up to 15 min at 80°C, indicating that the foaming properties of DFPE will withstand pasteurisation temperatures of foods. However foaming properties of DFPE will be reduced with high intensity thermal treatment compared to SPI.

3.3.3.3 Effect of heat treatment on emulsion activity index and emulsion stability index (EAI and ESI)

Figure 3.13 shows that the emulsion activity index (EAI) of DFPE was lower than that of SPI and was not affected by heat treatment compared to SPI. These differences in functionality correspond with increased FSH and hydrophobicity for SPI, whereas no effect was observed for DFPE (**Figures 3.6** and **3.8**).

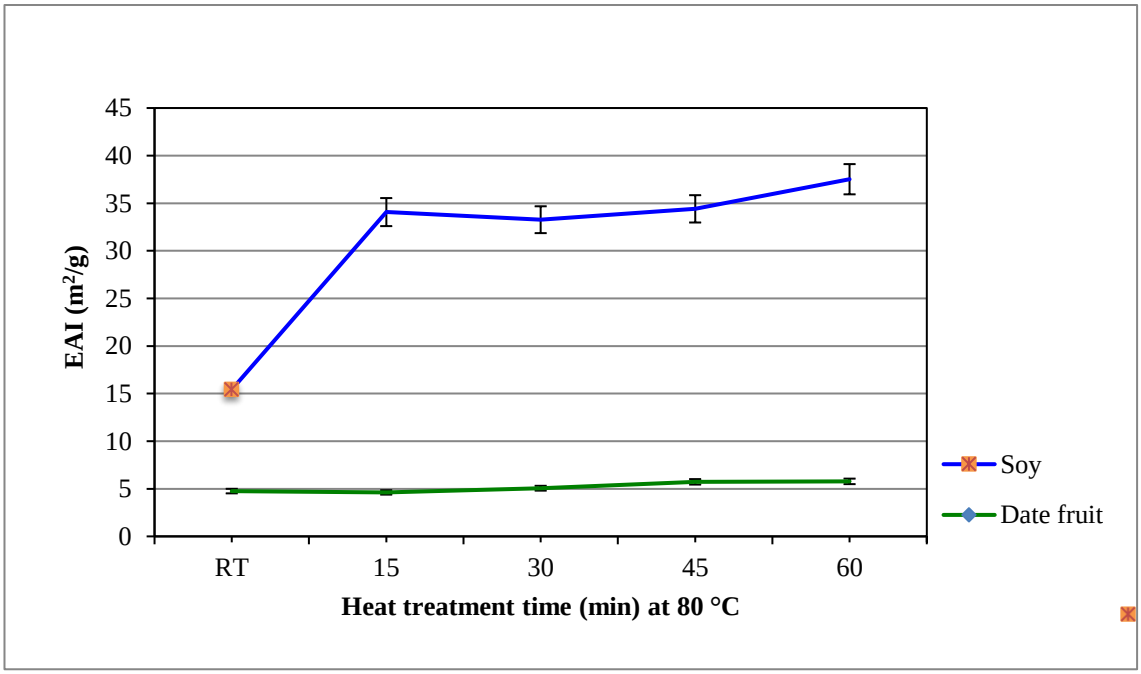


Figure 3.4: Effect of heat treatment on emulsion activity index of DFPE compared to SPI (1.13% protein).

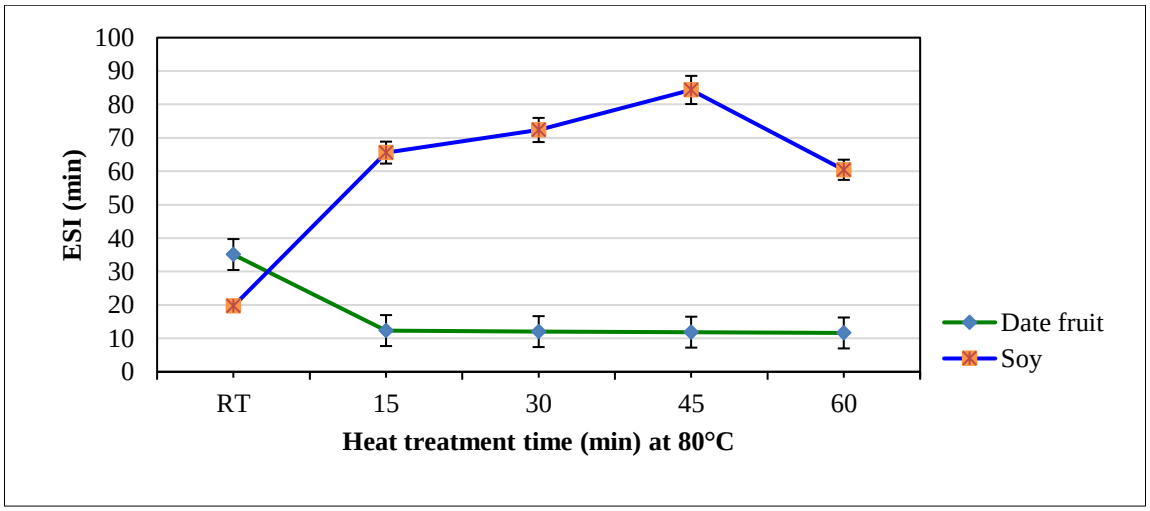


Figure 3.5: Effect of heat treatment on emulsion stability index of DFPE compared to SPI (1.13% protein).

Figure 3.14 shows that the ESI for DFPE was significantly ($p < 0.05$) higher than that of SPI for the non-heated samples at 35.08 versus 19.64. Heat treatment at 15 min and beyond significantly decreased the ESI for DFPE, which could be due to the formation of intra-molecular disulphide bonds at this heating intensity (**Figure 3.7**), negatively affecting its absorption at the oil/water interphase. Damodaran (2005) reported that excessive denaturation could impair the emulsifying properties by making the protein insoluble.

On the other hand, the ESI of SPI increased as a function of heat, probably because of the increased hydrophobicity of the protein (**Figure 3.8)** and increase in free SH groups (**Figure 3.6)**. Ahmed (2013) found that denaturation at 85°C for 30-150 min at pH 7.0 improved the emulsification properties for glycated cowpea protein isolate, which corresponded to increased hydrophobicity and an increase in free SH groups.

These results indicate that DFPE in solution is not as good an emulsifier as SPI, therefore will not be suitable for application in mayonnaise and pasteurised sauces, such as Hollandaise.

3.3.3.4 Effect of heat treatment on water separation of oil-in-water (O/W) emulsions

Figures 3.15 and **3.16** present the results of the effect of heat treatment of the protein on water separation of oil-in-water (O/W) emulsions. Water separation for SPI was significantly less than for DFPE confirming the results of EAI and ESI in the previous section. The degrees of water separation for both DFPE and SPI emulsions made with non-heated protein samples were less than for emulsions made with heat-treated samples. The water separation profile for DFPE correlates with that of EAI values (**Figure 3.13**). However, it does not correspond to that for SPI: according to **Figure 3.14**, the emulsion stability for SPI should improve as a function of heating, which was also reported by Nakamura et al., (2007).

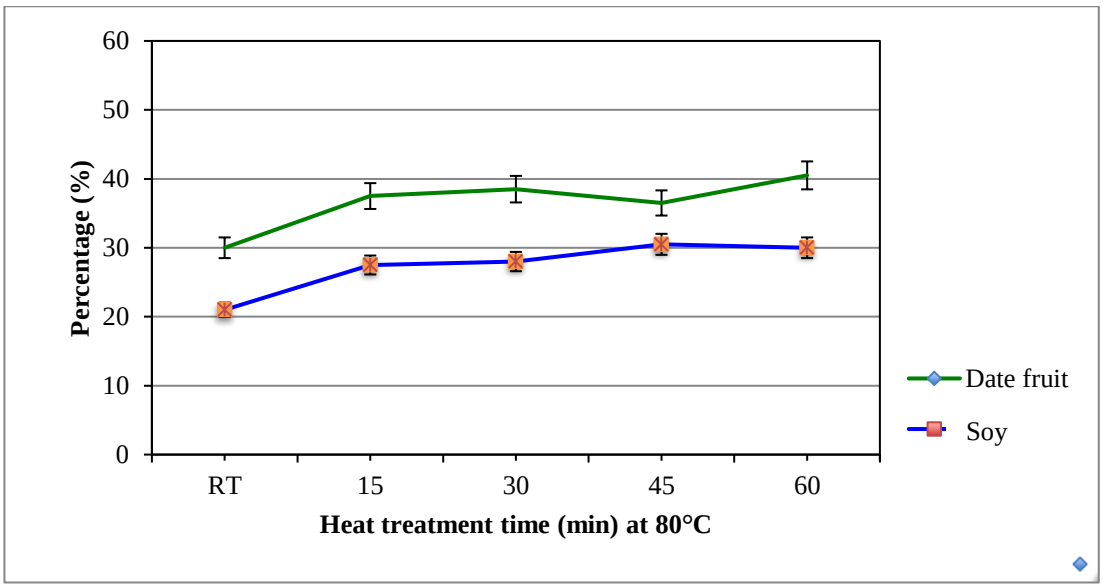


Figure 3.6: Effect of heat treatment on water separation of oil-in-water (O/W) emulsions for DFPE compared with SPI, 1.13 protein.

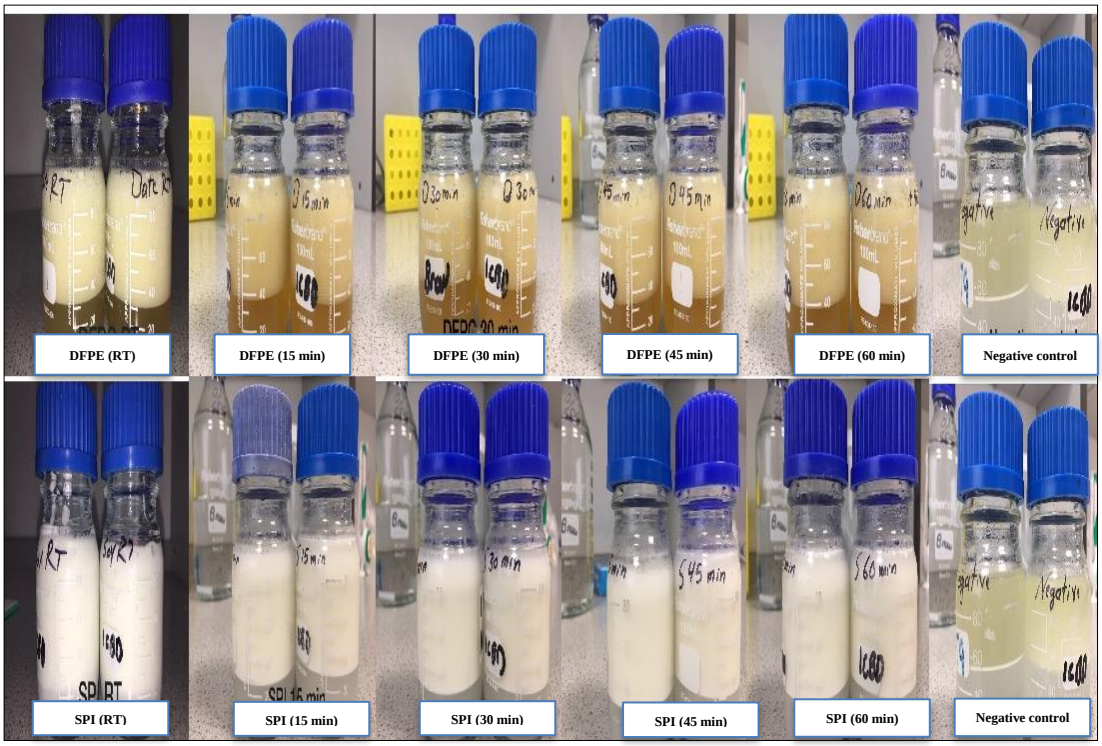


Figure 3.7: Effect of heat treatment of O/W emulsions for DFPE (upper samples) compared with SPI (lower samples), (1.13% protein).

These results confirm the results of the previous section that emulsifying ability of DFPE in solution is not comparable to that of SPI.

3.3.3.5 The water and oil absorption capacity of DFPE and SPI powders

In this section the WAC and OAC of DFPE powder that had not been subjected to heat treatment were compared to those of native SPI powder. **Figure 3.17** shows that SPI has a higher value for WAC (6.01 g/g) compared with that of DFPE (3.41 g/g sample). These results reflect those of Akasha (2014) who similarly found that WAC of SPI powder was (6.37 g/g) compared with that of date seed protein isolate (3.99 g/g). These findings are supported by Aletor et al., (2002) who reported that WAC in the range of (1.49 ml/g to 4.72 ml/g) is required to be functional in viscous food, such as soups and gravies. Thus, it can be assumed that the DFPE powder could be suitable for these types of food.

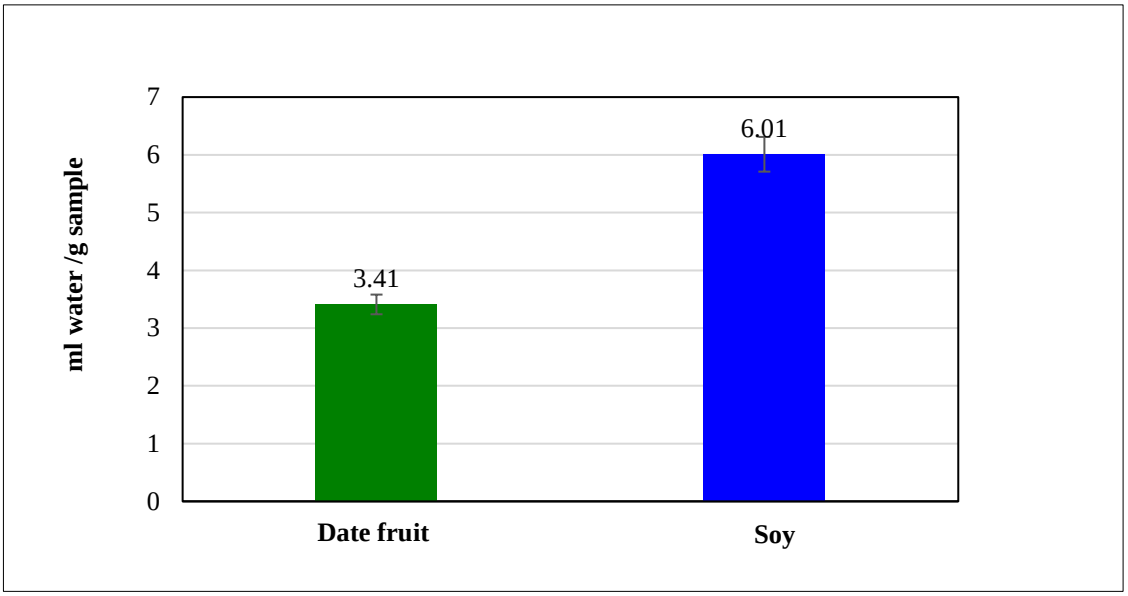


Figure 3.8: Water absorption capacity of DFPE compared to SPI, 2.58% protein.

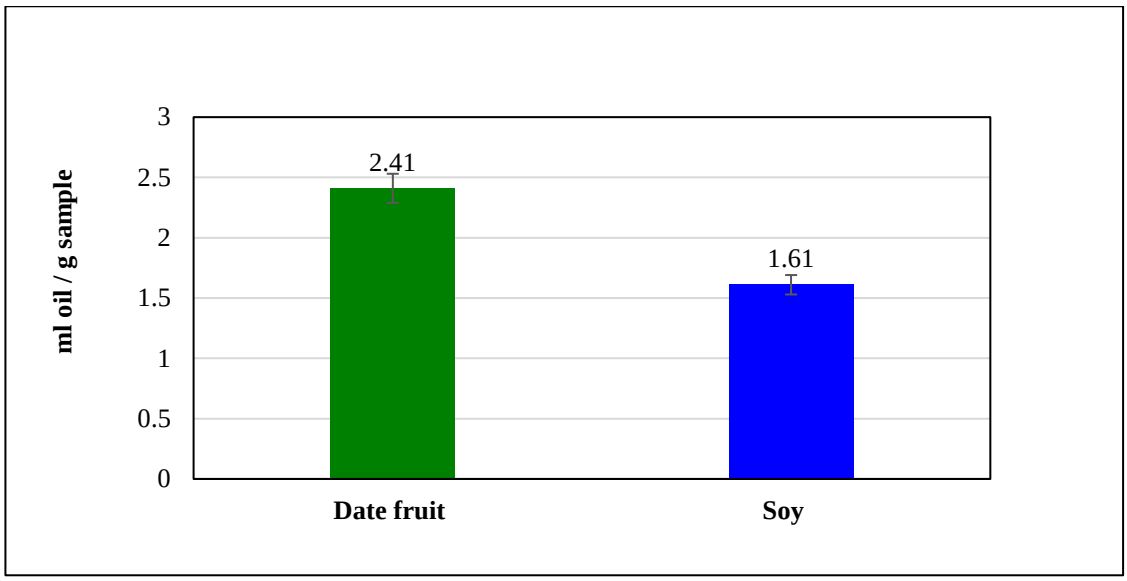


Figure 3.9: Oil absorption capacity of DFPE compared to SPI, 2.58% protein.

The value of OAC in **Figure 3.18** of DFPE (2.41 g/g) sample was higher than that of SPI sample (1.61 ml/g).

It should be considered that these results cannot be correlated with the hydrophobicity measurements (**Figure 3.8**) as the proteins were in solution, whereas results of **Figure 3.18** are for the proteins in the dry state. The value of (1.61 ml/g) for SPI in **Figure 3.18** is different than that reported by Gandhi (2012) of (8.3 ml/g), which could be due to difference in quality of soy protein ingredients tested. The functionality will be affected by the shelf life of the ingredients and the SPI that was tested in this study was 18 months which is close to the limit of its shelf life (2 years). In comparison, the SPI tested by Gandhi (2012) was freshly prepared. Nevertheless, the OAC for DFPE is approximately twice higher than that for SPI (2.41g/ml), confirming that it will be suitable for application in sausages and food batters. Soy protein isolate is widely used in the food industry in these food applications (Singh et al., 2008). These results confirm that DFPE has similar if not better functionality in this respect.

3.4 Conclusions

The physicochemical and functional properties of protein in date fruit protein are reported here for the first time.

Physicochemical properties

- The concentration of free and total sulphydryl groups were significantly less that for SPI, confirming the results of low cysteine of amino acid analysis results of DFPE (**Chapter 2**). The results indicate that DFPE is not as thermally stable as SPI, whilst considering the fact that DFPE had been subjected to heat treatment during the extraction process. The results indicate that DFPE at a concentration of 1.25% would withstand pasteurisation temperatures in the range of 15 min at 80°C but more intensive heat treatment will damage the protein. This physicochemical profile is mirrored by the corresponding decrease in functionality including decrease at longer heating times in solubility, foam capacity emulsion stability index and increased water separation in emulsions.
- The excellent solubility of DFPE could enable its application in baby food, beverages and drinks, such as smoothies with a fairly neutral pH, to enhance the protein content and replace soy protein. The solubility of DFPE at more acid pH range (3-5) still need to be investigated, but the present results indicate that DFPE solubility decreases at pH 6 compared to 7, implying that its application in acidic food formulations could be limited.

Functional properties

- DFPE could be used in foamed products provided that it is not exposed to excessive heat treatment. It could be applicable in baking where foaming capacity would enable creation of gas bubbles and stabilise the bubbles sufficiently until the baked product has been set (up to 15 min at 80°C).

- The DFPE powder showed water absorption capacity and oil absorption capacity in the range required for application in soups, cake batters and sausages and nutrition bars.
- On the other hand, it is evident that DFPE in solution is not as good an emulsifier as SPI and therefore will not be able to replace SPI in oil in water emulsions where the protein requires to be soluble in the water phase. This applies to products, such as mayonnaise and pasteurised fat containing sauces.

Further investigation is required to obtain a more knowledge of the physicochemical and functional properties of DFPE, which could not be conducted in this study due to the lack of availability of sufficient quantities of DFPE. Further investigation would include the effect of protein concentration, pH <6 and ionic strength on thermal stability and functional properties of DFPE in solution.

Further studies are also required to obtain an optimal balance between thermal treatment of date protein during the extraction process and the heat stability of the ingredient to withstand pasteurisation temperatures during food processing to maintaining its functional properties.

CHAPTER FOUR

Development of a process for preparation of a protein concentrate and characterisation of the protein

4.1 Introduction

The aims of research reported in this chapter are firstly the preparation of date palm fruit protein extract (DFPE) using three different extraction methods, in order to find the most efficient extraction method and improve that method to obtain the highest content of protein. The aim was to develop a process, which would be suitable to use in the food industry, which means that the process should not involve the use of toxic chemicals. The second aim was to characterise the electrophoretic profile of the chosen extract by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the third aim was to characterise the proteins in the extract by LC-MS/MS analysis.

4.1.1 Proteins in date fruit

To the researcher's knowledge there exists only one peer-reviewed publication on the characterisation of proteins isolated from date palm fruit, which was published by Ahmed et al., (1995a). In that study, the researcher selected nine varieties of date palm fruit samples from Oman, KSA, Iran and, the USA. The proteins were extracted using non-toxic chemicals, such as phosphate-buffered saline. The protein content of the extracts ranged from 0.06-0.14% and the protein yield ranged from 2.4-5.6% as measured. The main types of proteins in date fruit were albumins, which are water-soluble protein and globulins, which are soluble in salt solution. The study determined the protein content at all five stages of ripeness and found that the protein in the *Tamr* stage was the most abundant.

The protein profiles for dates from Oman, Iran and the KSA were similar and the complex mixtures of proteins in the molecular weight ranged from 12,000-72,000 MW (kDa), while dates from the USA had a small amount of protein and the protein profile had only one band, which was 30,000 MW (kDa), see **Figure 4.1.**

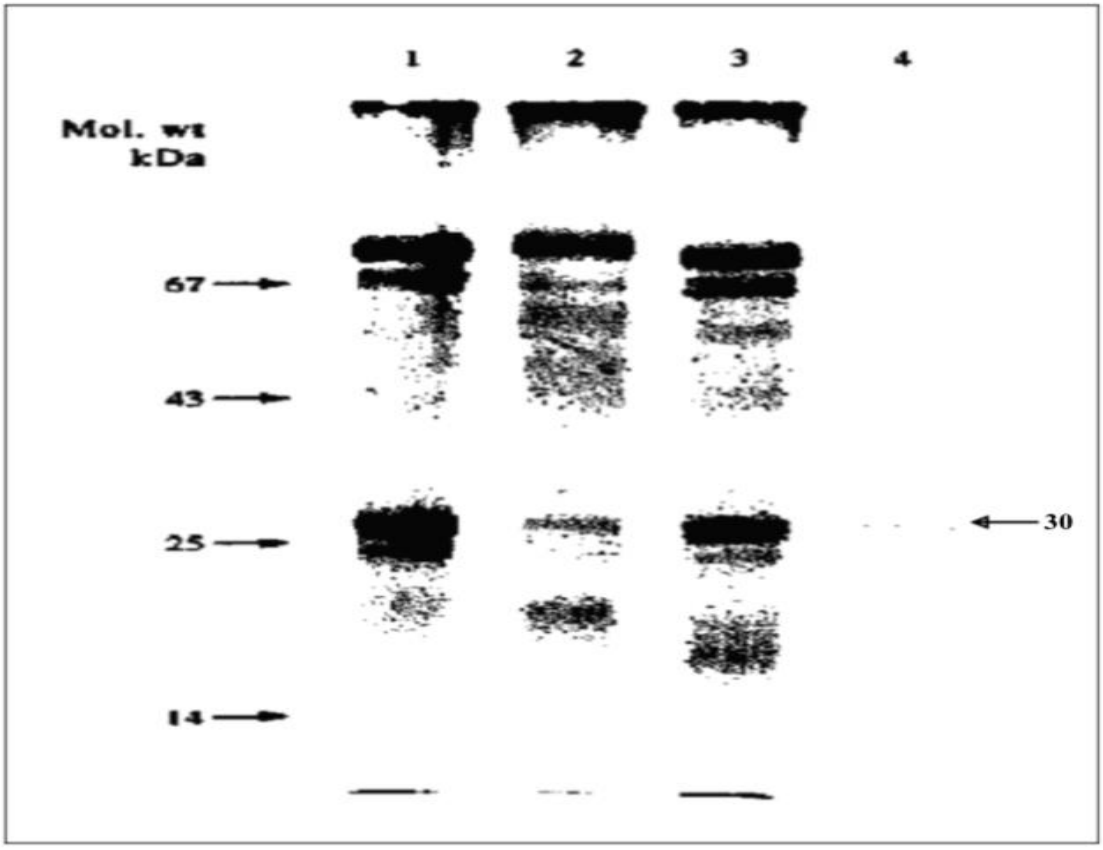


Figure 4.1: SDS profiles of proteins extracted from dates palm fruit from Oman (lane 1), Iran (lane 2), KSA (lane 3) and USA (lane 4) (Ahmed et al., 1995a).

The positions of the four standard proteins in the samples are outlined by arrows, these proteins are: bovine serum albumin (BSA, 67 kDa), ovalbumin (OVA, 43 kDa), prominent (30 kDa), chymotrypsinogen (25 kDa) and ribonuclease A (14 kDa).

A chapter in a book published by Al-Khayri et al., (2017) titled, "Date Fruit Proteomics During Development and Ripening Stages", describes the extraction of protein from date fruit using phenylmethylsulfonyl fluoride (PMSF) and mercapto-ethanol which are highly toxic substances and, therefore, not suitable for use in the food industry.

4.1.2 Principles of the analytical methods used in this chapter

The following section presents the principles of the methods used in this chapter.

4.1.3 Determination of DFPE protein concentration

The total protein content of DFPE (soluble and insoluble) was determined by using the Kjeldahl method (AOAC, 1995, method No. 988.05; Lynch et al., 1998), while the soluble protein content of DFPE was determined by using the Bradford assay, following Walker (2002).

Principle of the Kjeldahl method

The Kjeldahl method is the standard method of determining the nitrogen content of organic and inorganic substances. It has three basic steps: 1) Digestion, in which the sample is digested in sulfuric acid with a catalyst to digest the protein, thus releasing nitrogen from the sample, which is then converted into ammonia. 2) Distillation: in this step, the ammonia reacts with boric acid to form ammonium borate. 3) Titration: in this step diluted HCl (0.1N) is used to back-titrate the ammonium borate and the titre is used to calculate the total nitrogen content (Persson et al., 2008).

Limitations of the Kjeldahl

There are some limitations of Kjeldahl method, such as the technique is required a long time to carry-out. The method measures all nitrogen in the sample which could include that of nucleotides, hence it does not give a measure of the nitrogen only related to protein (People.umass.edu, 2019).

Principle of Bradford assay

The Bradford assay relies on the binding of the dye Coomassie-Blue G250 to protein. The colour of the dye changes from brown to blue depending on the concentration of the protein. The binding of the dye to protein causes a shift in the absorption spectrum maximum of the dye from 465 to 595 nm. This method is very reproducible and rapid, with the dye binding the blue colour in around two min and the colour being stable for one hour (Bradford, 1976).

Limitations of Bradford assay

The limitation of the Bradford assay is that it is very sensitive to some chemicals in the solutions, such as detergents and chaotropic agents. Bradford assay is depending on comparing the absorbance of the protein to that of a standard protein, such as bovine serum albumin. If the protein does not react to the dye (Coomassie-Blue which produces a characteristic blue colour upon binding to proteins in solution) in a similar way as the standard protein, it is possible that the concentration measured will be inaccurate (Okutucu et al., 2007).

4.3.7 Identification of proteins in date palm fruit protein extract using Liquid Chromatography Coupled with Tandem Mass Spectrometry (LC-MS/MS)

LC-MS/MS analysis of the date fruit protein extract (DFPE) powder was carried out by the School of Physics and Astronomy, School of Chemistry, University of Edinburgh, Scotland.

Statistical analysis

Triplicate determinations were performed for the analysis, using freshly prepared DFPE samples. The mean (±) and standard deviation (SD) values were calculated from these triplicates. A p-value of (<0.05) was considered statistically significant using independent

samples t-test SPSS version 10 for Windows (SPSS Inc., NY, USA).

4.1.3.1 Electrophoresis (SDS-PAGE) of DFPE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate and characterise proteins in order to determine their molecular weights, according to the methods of Havea et al., (1998) with some modifications.

In the electrophoresis process, the macromolecules are separated in an electric field. A method commonly used to separate proteins using electrophoresis uses a polyacrylamide as a support medium, and sodium dodecyl sulphate (SDS) in order to denature the proteins. This technique is known as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE is the most frequently used technique for analysing protein mixtures qualitatively. The strategy is based on the separation of proteins according to their size. Specifically, it is useful for observing the level of purification of protein; additionally, it is often used to determine the relative molecular mass of protein (Walker, 2002).

In polyacrylamide gels larger molecules migrate more slowly than smaller ones, because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of the polypeptides (Fuquay et al., 2011).

4.1.3.2 Identification of proteins in DFPE using Liquid Chromatography Coupled with Tandem Mass Spectrometry (LC-MS/MS)

Liquid chromatography–mass spectrometry (LC-MS) is a composite technique of chemical and physical separation. It can be used to identify a wide scope of biological molecules and the use of tandem MS and stable isotope internal standards enables extremely sensitive and precise examination. LC-MS equipment includes an interface

that conveys the separated combination from the LC column into the mass spectrophotometer (Pitt, 2009).

4.2 Materials

The *Shalaby* date, which is one of the most-consumed in KSA, particularly in the city of Al-Madinah Al- Munawarah, at the *Tamr* stage of ripening (brown stage) was chosen for this study because the fruit at this stage is fully ripe and is good for human consumption and storage. The protein content at this stage is in the range from 2.0 to 2.5%, while it is in the range from 5.5 to 6.4% at the *Kimri* stage (green stage). The concentration of protein decreases as the date ripens (Ashraf and Hamidi-Esfahani, 2011). The sample used in this research was purchased from a date farm from the city of Al-Madinah Al-Munawarah 41422, KSA (Al-Madinah Date Co).

Chemicals

Table 4.1: Chemicals obtained from Sigma Aldrich, UK

Chemicals obtained from Sigma Aldrich, UK	Catalogue number
Phosphate buffered saline	P4417
NaOH	1310-73-2
HCl	7647-01-0
Potassium sorbate ($C_6H_7O_2^-K^+$)	85520
Bovine serum albumin (BSA)	9048-46-8
Boric acid (BH_3O_3)	10043-35-3
Bradford reagent	B6916
Glacial acetic acid (CH_3COOH)	64-19-7
Coomassie-Brilliant Blue G250	6104-58-1
Laemmli, 2 x concentrate sample buffer containing 5% β-mercaptoethanol	S3401
β-mercaptoethanol	60-24-2
Trichloro-acetic acid (TCA, $C_2HCl_3O_2$)	76-03-9

Table 4.2: Chemicals obtained from Bio-Rad Laboratories, UK

Chemicals obtained from Bio-Rad Laboratories, UK	Catalog number
4-20% Bio-Rad Mini-protean precast gels	456-1094
A 10× Tris-Glycine/SDS running buffer	1610732

Table 4.3: Chemicals obtained from Thermo Fisher Scientific, UK

Chemicals obtained from Thermo Fisher Scientific, UK	Catalog number
Ethanol (C_2H_5OH)	64-17-5
o-phosphoric acid (H_3PO_4)	7664-38-2
SeeBlue Plus 2 Pre-Stained Standard	LC5925
The Pierce Protease and Phosphatase Inhibitor Mini Tablets contain aprotinin, bestatin, E-64, EDTA, and leupeptin.	A32959
Ultra-15 Centrifugal Filter Units, includes: two microcentrifuge tubes with cellulose membrane.	10092423

Table 4.4: Chemicals obtained from Thompson and Capper Ltd

Chemicals obtained from Thompson and Capper Ltd)	Catalog number
Silicone antifoam	AA00
Selenium (Se)	AA08

Table 4.5: Chemicals obtained from VWR Chemicals

Chemicals obtained from VWR Chemicals	Catalog number
Sulfuric acid 95% AnalaR NORMAPUR analytical reagent (H_2SO_4)	20700.320
Boric acid solution 4% (H_3BO_3)	192316H

4.3 Methods

4.3.1 Preparation of date fruit protein extract (DFPE)

The seeds were removed from the fruit and the fruit was then chopped into small pieces with a kitchen knife. The fruit was stored in polythene bags in a freezer at - 40°C awaiting further analysis.

Three methods were used for preparation of protein from date palm fruit, which are described in the following sections:

Method 1

This procedure was based on that described by Ahmed et al., (1995a) with some modifications as presented in **Figure 4.2**. The steps from 1 to 4.2.11 were followed as outlined in **Figure 4.2**. 100 g of date palm fruit was soaked in 200 ml of phosphate buffered saline (PBS, pH7.4) and 0.1% (w/v) potassium sorbate ($C_6H_7O_2^-K^+$) (buffer A)

at RT for 24 h. The soaked dates were crushed by a kitchen blender (BL370 series, Kenwood Ltd, UK) for 2 min. The mixture was stirred for 24 h at RT using a magnetic stirrer (Stuart® stirrer SB 162, Bibby Scientific Ltd, UK) and then centrifuged at 6000 rpm for 20 min at RT using (Beckman Coulter, Avanti J-26 XP centrifuge with a JA 25.50 rotor, Beckman Coulter, USA). The next series of steps on the resulting supernatant (**4.1** in **Figure 4.2** was conducted to optimize extraction of proteins and consisted of repeat steps of precipitation of protein at pH 4, centrifugation and resuspension of pellet at pH 7. The pellet **(step 4.2.2)** was resuspended in water to optimize extraction followed by acid precipitation, centrifugation and resuspension of the resulting pellet at pH 7.

The supernatants in steps (**4.1.4**, **4.2.2**, **4.2.8** and **4.2.9**) were then combined and freeze-dried. The final powder was stored at -20°C in polythene bags for further analysis.

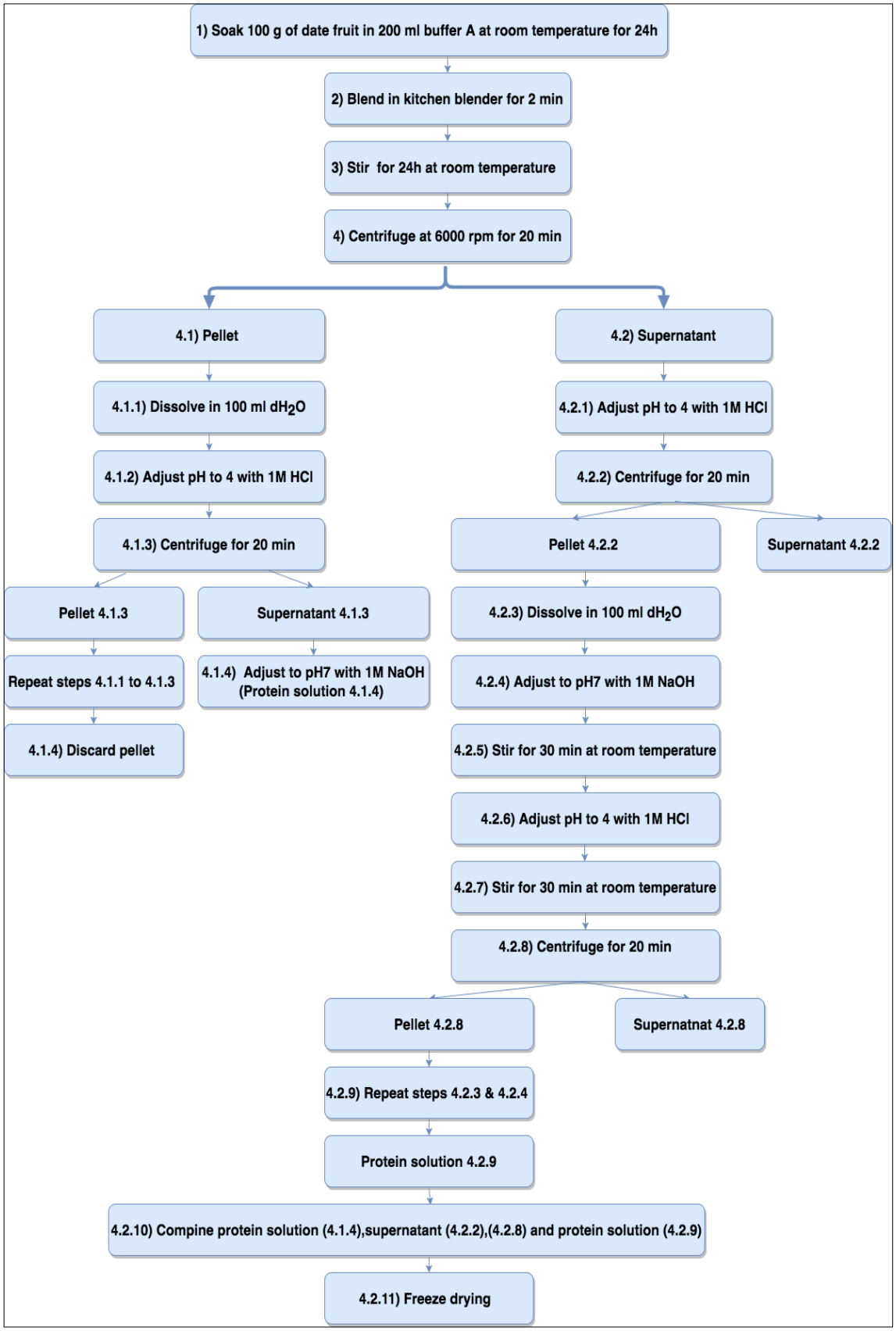


Figure 4.2: Method 1 used for extraction of date protein, based on the method of Ahmed et al., (1995a).

Method 1.1

The chosen method used for extraction of protein was based on method 1, with some modifications to obtain the highest content of protein from the sample, as shown in **Figure 4.3**.

Method 1.2

Proteases in the fruit lead to the digestion of the protein; thus, two approaches were followed to inactivate the proteases during the extraction procedure of method 1.1: (A) application of heat and (B) addition of protease and phosphatase inhibitor.

A. The fruit was heated in step 2 of method 1.1 at 80°C for 30 min.
B. Protease and phosphatase inhibitor were added to the mixture to a concentration of 0.1 mg/ml**.**

The efficiency of inactivation by proteases was determined by measurement of the protein yield after extraction using method 1.2, compared to the yield without used of proteases.

A. Heat treatment:

The first modification in method 1.1 was heating the fruit slurry in a water bath at 80°C for 30 min at step 2, while in method 1 the fruit slurry was not heated. The second modification for method 1.1 was cooling the fruit slurry in ice at 40°C then centrifuging the sample at 10000 rpm at 4°C, while in method 1 the sample was centrifuged at 6000 rpm at room temperature. The third modification in method 1.1 was adjusting the pH to 4 using 20% acetic acid, while in method 1 using 1M HCl. The fourth modification in method 1.1 was that all pellets were dissolved in 100 ml dH_2O and heated at 80°C for 30 min, then centrifuged and the final pellet in method 1.1 was then freeze-dried, while in method 1 the pellet was freeze-dried without heating.

B. Protease treatment:

The steps of this method, as presented in **Figure 4.3,** were similar to those in method 1.1, but the main difference was adding 1mg protease and phosphatase inhibitor to 10 ml of the supernatant at step **5.2.5.1.**

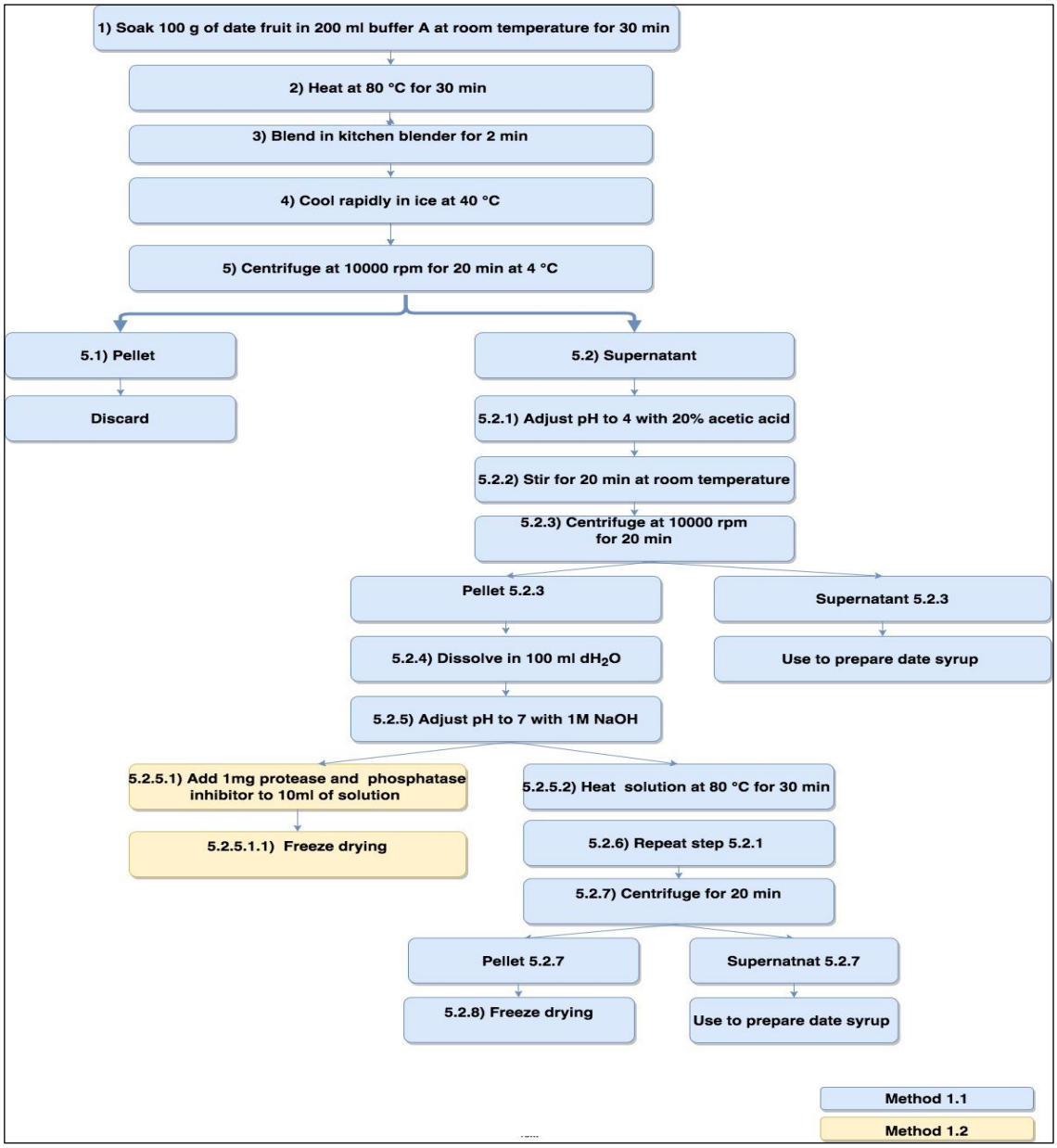


Figure 4.3: Methods 1.1 and 1.2 for extraction of date protein, based on the method of Ahmed et al., (1995a). The blue colour represents method 1 and the yellow is method 2.

Method 2

This procedure was based on the procedure described by Elleuch et al., (2008), with some modifications (**Figure 4.4)**. Fresh date palm fruit (50 g) were mixed with 600 ml of hot dH_2O and stirred for 10 min at room temperature. The supernatant was discarded after centrifuging at 6000 rpm for 20 min at room temperature, while the pellet was washed with 300 ml of dH_2O at 40 ℃ and centrifuged again as above. This operation was repeated five times. After a final washing with dH_2O, the supernatant was concentrated using Ultra-15 centrifugal filter units, while the pellet was discarded. The retentates were freeze-dried. The final powder was kept in a freezer in polythene bags awaiting further analysis.

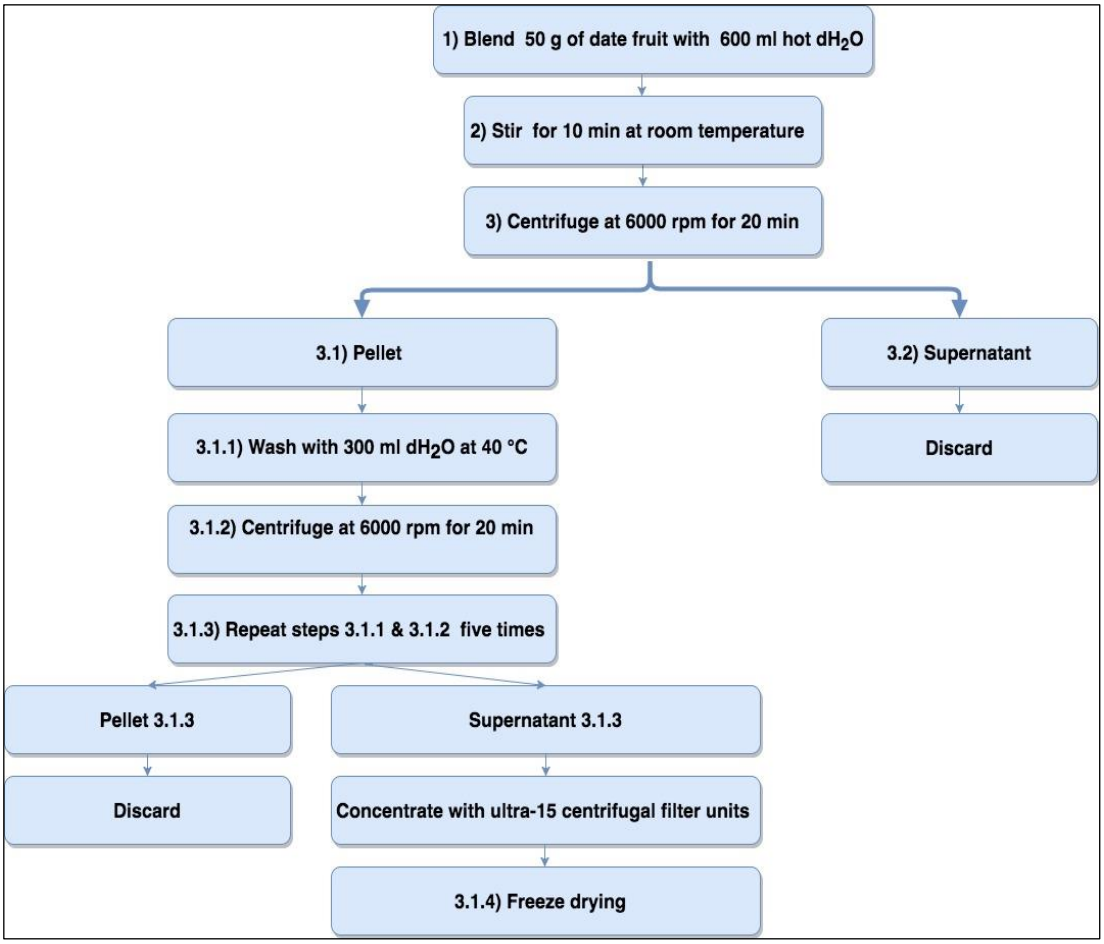


Figure 4.4: Process of method 2, based on Elleuch et al., (2008).

Method 3

This method is outlined in **Figure 4.5** and based on the method employed by L'Hocine et al., (2006) to prepare of soy protein concentrate, with a slight adjustment.

The steps from 1 to 4.2.6 were followed as outlined in **Figure 4.5.** The final powder was kept in a freezer in polythene bags awaiting further analysis.

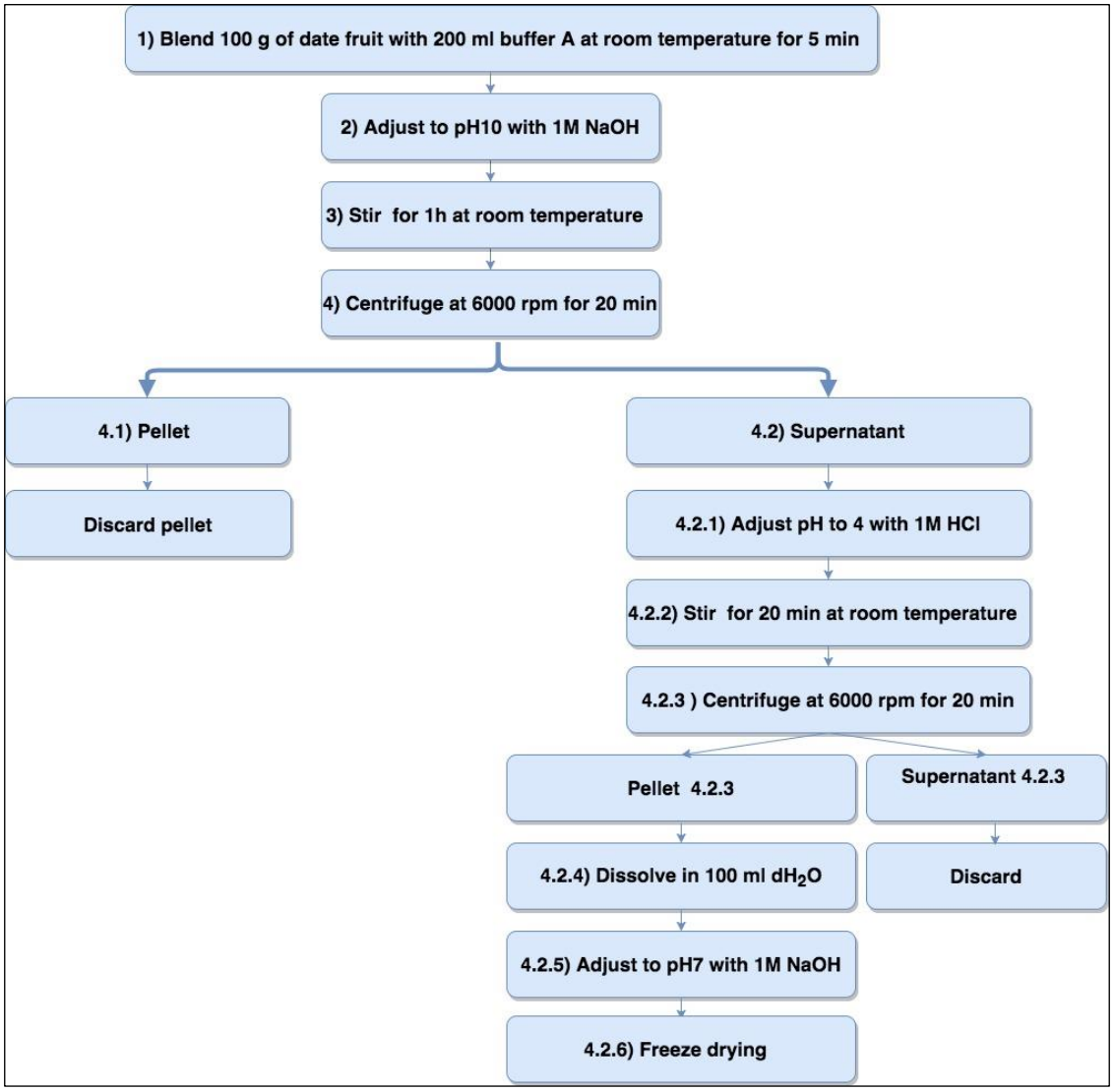


Figure 4.5: Process of method 3, based on L'Hocine et al., (2006).

The differences between methods 1 and 3 are as follows. In method 3 the pH of the mixture was adjusted to 10 using 1M NaOH before centrifuging at step 2, while it was not adjusted in method 1 at the same step. The pellet in step 4.1 was discarded in method 3, while in method 1 the pellet was dissolved in 100 ml dH_2O. In method 3 the supernatant

at step 4.2.3 was discarded, while in method 1 all supernatants were combined and freeze-dried. The last difference was that the supernatant at step 4.2.5 of method 3 was freeze-dried, while in method 1 it was adjusted to pH 4 then centrifuged and freeze-dried.

4.3.2 Large scale preparation of date fruit protein

To prepare larger quantities of protein extract, method 1.1 (**Figure 4.3)** was selected and carried out as shown in the figure below.

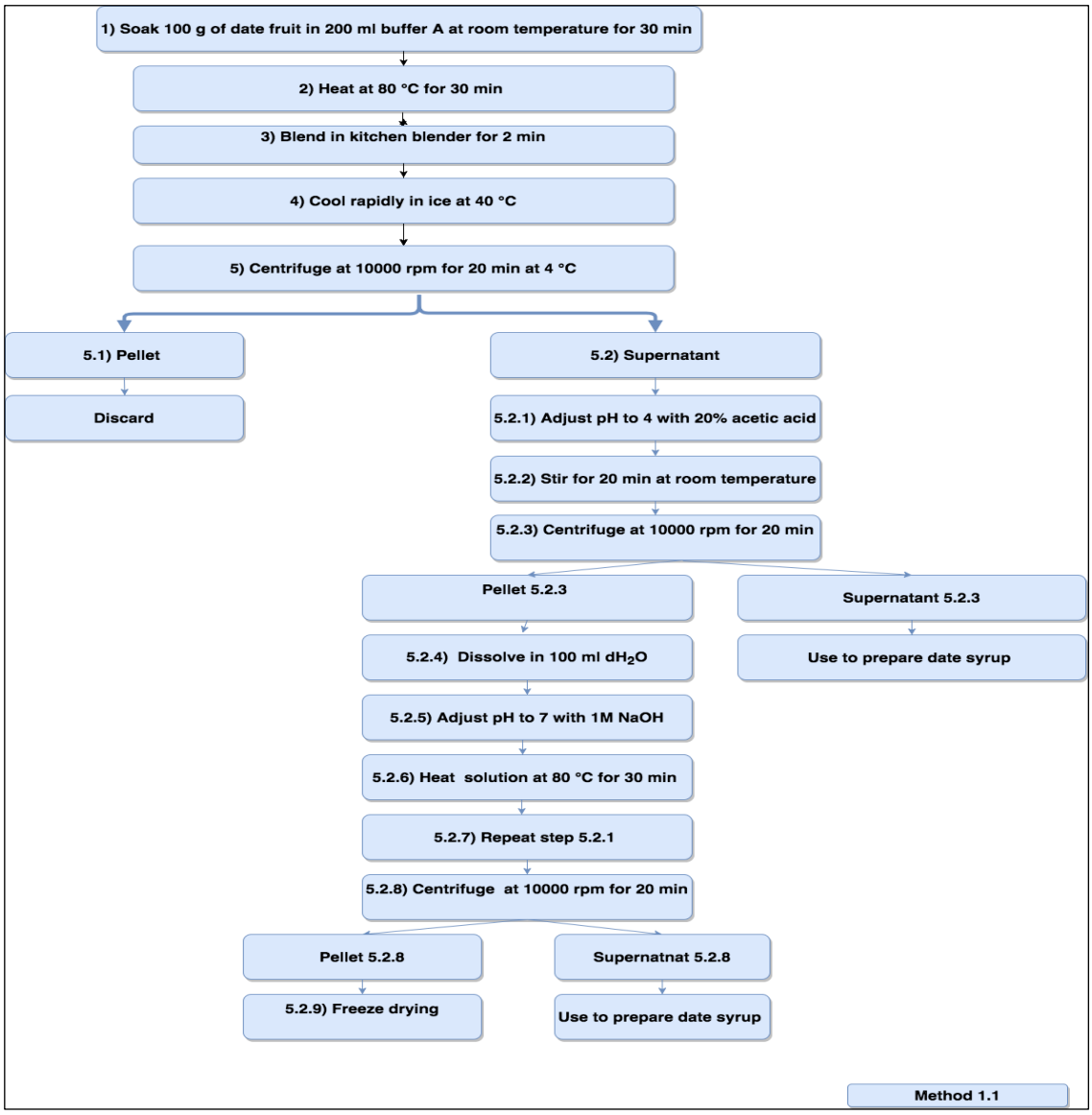


Figure 4.6: Method 1.1 for preparation of larger quantities of protein extract

4.3.3 Preparation of date fruit syrup

Date fruit syrup was prepared by evaporated the water from the supernatant (**method 1.1**, **Figure 4.3**, **step 5.2.3** and **5.2.7**) using (VonShef) food dryer at 40°C for 12 hours. The syrup was stored in a freezer at - 40°C awaiting further analysis.

4.3.4 Protein determination

The total protein content of DFPE (soluble and insoluble) was determined using the Kjeldahl method (AOAC, 1995, method No. 988.05; Lynch et al., 1998). Samples of 0.05-0.1 g were weighed into a digestion flask and two tablets of silicone antifoam (each one containing 0.97 g sodium sulfate and 0.03 g silicone antifoam) and one tablet of KJELTAB S catalyst (containing 5 g potassium sulfate and 5 mg selenium (Se) were added. Concentrated sulfuric acid (12 ml) was carefully added to each digestion flask. Sulphuric acid functions to digest the protein, thus releasing nitrogen from the sample that is in turn converted into ammonium ions. The flasks were then transferred to a digestion unit (TecatorTM digester system 20, Foss-UK) where the samples were heated to 440°C until the samples became clear (approximately one hour). The flasks were heated for a further 10 min before the digestion flasks were cooled to RT and transferred to a distillation unit (KjeltecTM 8100 distillation system, Foss-UK). Water (70 ml) and 40% (w/v) NaOH (50 ml) were added to each flask and the samples distilled for 3 min for each sample to release ammonia from the sample. The ammonia was received into a cleaned and dried beaker containing 25 ml of boric acid (4%). The ammonia reacts with the boric acid to form ammonium borate.

Dilute HCl (0.1N) was used to back-titrate the ammonium and the titre used to calculate the total nitrogen content. The protein content was calculated as a percentage of the dry weight of the sample by multiplying the total nitrogen content by a conversion factor of

6.25. This value of 6.25 is assigned to convert nitrogen content in pulses and cereals to protein (Hall and Schonfeldt, 2013).

Determination of soluble protein

A colorimetric protein assay was used to determine the concentration of soluble protein according to the Bradford assay, as described by Walker (2002), with some adjustments. Bovine serum albumin (BSA) was used to prepare a standard curve by using serial dilutions of 5, 10, 25 and 50 mg of BSA from the initial concentration of 1 mg/ml.

100 mg of dried DFPE was dissolved in 10 ml of dH_2O; the sample was stirred for 20 min and centrifuged at 6000 rpm for 20 min at RT to remove insoluble protein. The supernatant was diluted 1:1, 1:2, 1:5 and 1:10 with dH_2O. 1 ml of each BSA standard solution and 1ml of diluted supernatant from each sample, respectively was mixed with Bradford reagent (3 ml) and incubated for 10 min at RT. The absorbance of samples was measured at 595 nm using a UV-VIS spectrophotometer (Novapec II Biochron Ltd., UK). The standard curve was calculated using the equation Y = 0.0108x+0.0691, where y is OD (A) and x is concentration (mg/ml). The protein content of the date samples was calculated according to the standard curve equation. A solution of 1ml of dH_2O and 3ml of the Bradford reagent was used as a blank.

4.3.5 Protein yield calculation

Protein yield of DFPE was calculated using the following equation used by Selling et al., (2013):

$$Protein\ yield\ (\%) = \frac{\%\ \text{Protein content per weight of dried pellet}}{\%\ \text{protein content per dry weight of starting material}} * 100$$

4.3.6 Electrophoresis (SDS-PAGE) of DFPE

The (SDS-PAGE) analysis was performed based on the method described by Wu and Hojilla-Evangelista (2005).

Preparation of samples for electrophoresis (SDS-PAGE)

50 mg, 100 mg and 150 mg /ml of date fruit protein were dissolved in dH_2O and stirred for one hour. Insoluble protein was separated from the supernatant by centrifuging the suspension for 10 min at 6000 rpm and then the supernatant was used for the experiment.

Electrophoresis (SDS-PAGE) protocol

The analysis was performed using the method of Wu and Hojilla-Evangelista (2005) with some modifications, using 4-20% Bio-Rad Mini-protean precast gels. SDS-PAGE gels were run under denaturating conditions. The sample was prepared by mixing equal amounts of protein solution 500 µl and 500 µl of Laemmli, 2 x concentrate sample buffer with 5% β-mercaptoethanol as a reducing agent and vortexed for 1 min using a Lab Dancer (IKA, Germany). The samples were centrifuged at 2000 rpm for 1 min in a microfuge tube and the supernatant was heated in a water bath at 70°C for 10 min. A 10× Tris-Glycine/SDS running buffer was diluted 1:10 with deionized H_2O before use. The SeeBlue Plus2 Pre-Stained was used as molecular weight marker control. The wells were loaded with 30 µl of sample or 10 µl of marker and the gels were run in the running buffer at 180V for approximately 50 min or until the dye front reached the bottom of the gels. After completion of electrophoresis the gels were carefully removed from the plastic frames and rinsed with deionized H_2O and stained with a colloidal Coomassie-Brilliant Blue G250 solution containing 5% (w/v) aluminum sulphate hydrate, 10% (v/v) ethanol, 0.02% (w/v) Coomassie-Brilliant Blue G250 and 8% (v/v) o-phosphoric acid and then left overnight on a shaker. The gels were rinsed with water and kept in 10% ethanol and

2% phosphoric acid (de-staining solution) for 2 hours on a shaker. The gels were rinsed with H_2O until the background stain was removed and became clear. The SDS-PAGE gels were scanned using a BIO-RAD Molecular imager ChemiDoc XRS^+ imaging system and analyzed using GelAnalyzer 2010a gel electrophoresis analysis software to estimate the molecular weight of the proteins (https://en.freedownloadmanager.org/users-choice/Bio_Rad_Image_Lab_4.0.html).

Preparation of SDS sample with trichloro-acetic acid (TCA)

To purify the protein from contaminating fibres, trichloro-acetic acid (TCA) precipitation was carried out. First, 250 µl of fib% TCA was mixed with 1ml of DFPE. This was then incubated for 30 min on ice and centrifuged for 30 min at 6000 rpm at RT. Next, the pellet was mixed with 500 µl of cold acetone, vortexed briefly, and centrifuged at 4°C for 15 min. The pellet of protein was left to air dry and then dissolved in 1 ml dH_2O. Finally, 200 µl of 1 M NaOH and 200 µl of the protein solutions were added to equal volumes of reducing sample buffer and heated in a water bath at 80°C for 5 min, then centrifuged for 20 min. This was mixed and loaded into the gel.

4.4 Results and Discussion

4.4.1 Determination of total protein content by Kjeldahl method

Figure 4.7 represents the protein content of DFPE as determined by the Kjeldahl method, using the three extraction methods (**Section 4.3.1**), compared to the protein yield.

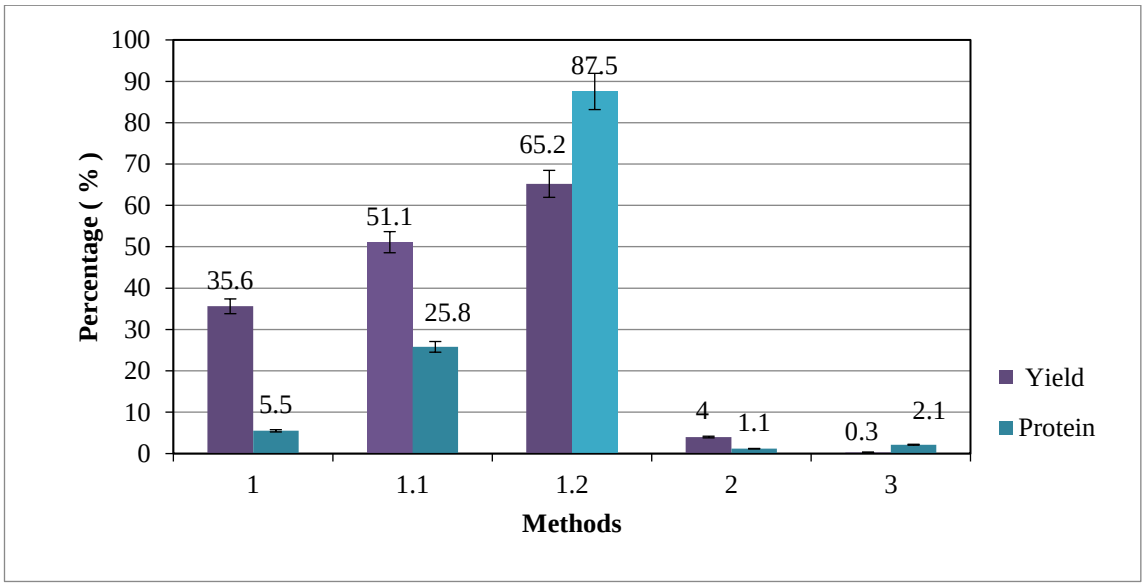


Figure 4.7: Protein content as determined by the Kjeldahl method and yield.

The use of method 1 resulted in a powder with protein concentration of 5.5% and protein yield of 35.6%. The improved method (1.1) resulted in a powder with protein concentration of 25.8% and a yield of 51.1%. This demonstrates that thermal treatment at pH 7 before the acidification step increased the quantity of precipitated protein. This could be attributed to the denaturation of globular proteins at pH 7, which enhances precipitation upon acidification at the iso-electric pH region (Boulet et al., 2000; Eromosele et al., 2008). Another factor of heat treatment could be that the contaminating proteases were inhibited leading to increased protein yield. This is supported by evidence that addition of protease inhibitors in **method 1.2** resulted in a powder with concentration of 87.5% protein and yield of 65.2%. The effect of protease inhibitors demonstrates that proteases negatively affect the protein yield. However, as protease inhibitors are not fit

for human consumption, the preferred method of extraction is method 1.1, where protease activity is inhibited by the thermal treatment.

Method 2 resulted in a pellet with protein content of 1.1% and yield of 4.0%, in which the solution was concentrated using ultrafiltration with Ultra-15 centrifugal filter units. The low yield could be attributed to protease activity.

Method 3 resulted in the lowest protein concentration (2.1%) and yield (0.3%), which could also be due to protease activity.

4.4.2 Recovery of materials in starting material of date fruit

Table 4.6: Recovery of materials in starting material of date fruit.

Material	g	% of date starting material	fibre (g)	% of date fibre	protein (g)	% of date protein	carbohydrate (g)	% of date carbohydrate
DFPE	59	4.2	1.8	1.6	14.7	49	30.3	2.7
Date syrup	800	57.2	13.7	12.2	8	26.7	792	70.7
Waste	541	38.6	96.5	86.2	7.3	24.3	297.7	26.6
TOTAL	**1400**	**100**	**112**	**100**	**30**	**100**	**1120**	**100**

The yield of different fractions extracted from the date fruit is shown in **Table 4.6**, based on proximate analysis of DFPE and date syrup discussed in (**Chapter 2**, **Section 2.3.1)**. The fraction of DFPE that was extracted from date starting material was 4.2%. The yield of fibre, carbohydrate and protein from starting material of DFPE are 1.6, 30.3 and 49% respectively. The highest recovery of material in DFPE was carbohydrate (57.2%, **Figure 4.6**), which is attributed to the high content of sugar in the starting material (80.7% carbohydrates in dry dates which consists mostly of sugar. The protein recovery in DFPE was 25.8%**,** which could have been higher if protease and phosphatase inhibitor were used**.** Concentration of the supernatant that resulted from the extraction process resulted in a date syrup containing 70.7% sugar). Date syrup was prepared to get all benefits from

the resulting waste. Waste resulting from the extraction process was 38.6% with a fibre content of 86.2%.

4.4.3 Electrophoresis (SDS-PAGE)

Electrophoretic patterns of different concentrations of DFPE and SPI are shown in **Figure 4.8**. Distinct protein bands in DFPE are detected at 16, 64, 100 and 149 kDa, with minor bands at 5, 20, and 36 kDa. There is a diffuse region of stained protein bands between 36 and 55 kDa. It was difficult to obtain clear peptide profiles for DPFE, despite attempts to optimize sample preparation, such as precipitation by trichloro-acetic acid (TCA). The appearance of diffuse bands could be due to the presence of fibres or carbohydrates. Ahmed et al., (1995a) carried out SDS-PAGE electrophoresis on different varieties of date fruit and they found two prominent bands at 72 kDa and 30 kDa. Here, the extraction of an extended range of proteins is described for the first time. For comparison purposes, SPI has visible bands ranging between 4 and 150 kDa in lanes 8, 9 and 10 of **Figure 4.8**.

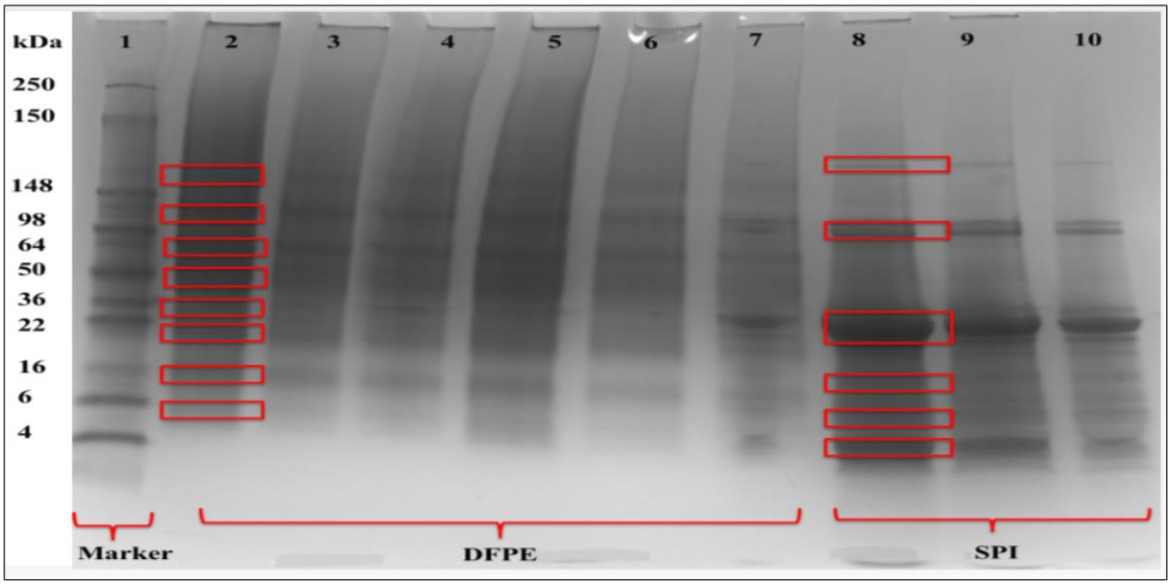


Figure 4.8: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions for different concentrations of DFPE. Lane 1 represents the molecular weight markers and the concentrations of DFPE in lanes 2 and 3 are (150 mg/ml), lanes 4 and 5 (100 mg/ml) and in lanes 6 and 7 (50 mg/ml) while the concentrations of SPI in lanes 8, 9, and 10 are (150, 100, and 50 mg/ml) respectively.

4.4.4 Identification of proteins in date palm fruit protein extract using Liquid-Chromatography -Tandem Mass Spectrometry (LC-MS/MS)

Principles of the LC-MS/MS analysis

Liquid chromatography (LC) is a separation method used to isolate the components in solution of a liquid mixture. The separation of the compounds is based on affinity towards the stationary phase. The compounds which have more affinity with stationary phase will be eluted slowly and compounds with less affinity with stationary phase will be eluted fast (Parasuraman et al., 2014). As the separated compounds elute from the column, they are injected into a mass spectrometer." A mass spectrometer generates multiple ions from the sample under investigation, it then separates them according to their specific mass-to-charge ratio, and then records the relative abundance of each ion type. The mass spectrometer consist of: an ion source, which can convert gas phase sample molecules into ions; a mass analyzer, which sorts the ions by their masses by applying electromagnetic fields and a detector, which measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present" (Ju-Seop Kang, 2011). The NCBI database and Mascot version 2.4 (Matrix Science Ltd, UK) were used to identify proteins based on the results of Liquid-chromatography-Coupled Mass Spectrometry (LC-MS/MS) for DFPE. The molecular weight search (MOWSE score) probability-based algorithm was used to identify the proteins, with at least two peptides being matched for every predicted peptide map for the protein sample, as presented in **Table 4.7**. Numerous candidate proteins were identified for each of the protein hits. Two standards were used to obtain an accurate identification of the proteins, the condition and the MOWSE score, in which the identification depended on at least two peptides being matched to the known peptide map of a protein. The MOWSE score is a process for identifying proteins based on the molecular weight of the peptides obtained from

proteolytic digestion of the protein sample (Abdurrahman, 2014). The method described by Pappin et al., (1993) for expression of the accurate identification a higher number was used. The hit number (HN) is a rough indicator of protein abundance in the sample, with a lower hit number indicating a more abundant protein. The score number reflects the combination of the scores of all observed mass spectra that can be matched to known amino acid sequences in a database. A higher score indicates a more confident match, and the number of peptide matches compared to the total number of peptides produced. In order to determine which peptide sequence in a database of protein sequences gives the best match, specific software is used. Various scoring algorithms have been devised to decide which peptide sequence best matches a given MS/MS spectrum (Cattrell, 2011). Searching of this database revealed about 550 hits. The proteins were screened to rule out any hits created from contamination, such as clusters, trypsin autolysis peptides, matrix molecules, skin keratins, and hair. **Table 4.7** presents the results of proteomic analysis. Ninety-nine of the hits have been selected based on MOWSE scores higher than a hundred; the rest of the hits with MOWSE scores of less than 90, have been removed. The selection was classified based on hit number (HN), the protein's name, the (MOWSE score), protein molecular weight (MW) and number of peptide matches compared to total number of peptides produced. These proteins were categorised into 11 various categories, depending on their functional categories, as used previously by Bevan et al., (1998).

Table 4.7: Selection of 99 proteins with highest MOWSE scores resulting from proteomic analysis with LC-MS/MS. "-like" means similar but not confirmed.

HN	Protein name	MOWES Score	MW (kDa)	Protein matched
Functional category 1: Metabolism: Amino acids/ nitrogen and sulphur/ nucleotides/ phosphate/sugars and polysaccharides/ lipid and sterol cofactors				
17	Isoflavone reductase-like protein	274	33855	9/9
26	SCAR-like protein	223	252482	9/9
11	Adenosylhomocysteinase	387	53806	8/8
49	NADP-dependent malic enzyme	163	71308	8/7
50	SCAR-like protein	161	258611	8/8
33	Nucleoside diphosphate kinase B-like	201	16427	7/7
37	Luminal-binding protein 5	191	73552	7/6
38	Actin-3-like	185	41948	7/7
41	γ-aminobutyrate transaminase 1, mitochondrial, partial	174	55850	7/7
57	Actin-101-like	155	41804	7/7
73	Puromycin-sensitive aminopeptidase isoform X1	133	111774	6/6
110	18.1 kDa class I heat shock protein-like	101	11792	6/6
36	ligase-like	195	52306	5/5
44	Aspartate aminotransferase, chloroplastic-like	168	50098	5/5
58	Aminopeptidase M1-like	154	101041	5/5
72	Ketol-acid reductoisomerase, chloroplastic-like	133	64534	4/4
62	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 1-like	148	85024	3/3
102	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 1-like	108	85135	3/3
105	Probable glutathione S-transferase GSTF1	101	24188	3/3
108	Monodehydroascorbate reductase-like	101	46745	3/3
94	Vacuolar protein sorting-associated protein 32 homolog 2	113	18961	2/2
Functional category 2: Energy: Glycolysis/gluconeogenesis /pentose phosphate/ TCA pathway/ respiration/fermentation/E-transport/photosynthesis				
5	ATP synthase subunit β-, mitochondrial-like	497	59650	11/11
16	Phosphoenolpyruvate carboxylase 2-like isoform X1	274	110548	11/11
18	Phosphoenolpyruvate carboxylase, housekeeping isozyme	271	111016	10/10
39	β-fructofuranosidase, insoluble isoenzyme 3-like	184	53513	10/9
13	β-fructofuranosidase, insoluble isoenzyme 3-like, partial	329	65112	14/13

19	Fructose-bisphosphate aldolase cytoplasmic isozyme-like	269	39047	7/7
23	Fructose-bisphosphate aldolase cytoplasmic isozyme	241	38837	5/5
32	Enolase-like	203	48373	5/5
79	phosphoglucomutase, cytoplasmic 2	128	63769	5/5
97	Fructose-bisphosphate aldolase cytoplasmic isozyme-like	111	38948	3/3
101	ATP-citrate synthase β-chain protein 1-like	108	66609	3/3
86	Malate dehydrogenase-like	121	35771	2/2
1	Sorbitol dehydrogenase-like	1089	39134	44/43
3	Glyceraldehyde-3-phosphate dehydrogenase 3, cytosolic	510	37102	15/15
63	Isocitrate dehydrogenase [NAD] catalytic subunit 5, mitochondrial	148	40784	3/3
*Functional category 3: Cell growth/division/*DNA synthesis/replication/recombination/repair/cell cycle/cytokinesis/growth regulators				
71	Cell division cycle protein 48 homolog	135	90575	6/6
65	DNA-damage-repair/toleration protein DRT102	147	34292	3/3
90	Cell division cycle protein 48 homolog	117	90635	5/5
55	Tubulin β-chain-like	157	50690	4/4
Functional category 5: Protein synthesis: Ribosomal proteins/translation factors/ translation control/tRNA synthases				
61	Eukaryotic initiation factor 4A-15-like	150	47273	7/7
98	Eukaryotic initiation factor 4A-8-like	110	47312	6/6
Functional category 6: Protein destination and storage: Folding and stability/ targeting / modification/ complex assembly/ proteolysis storage protein				
4	Heat shock cognate 70 kDa protein	501	71558	14/13
6	Heat shock cognate 70 kDa protein	483	71476	14/12
7	Heat shock cognate 70 kDa protein	465	71535	12/11
56	16.9 kDa class I heat shock protein	156	18002	8/8
42	17.8 kDa class I heat shock protein	170	17341	7/7
35	Peptidyl-prolyl cis-trans isomerase	200	18496	6/6
40	Heat shock 70 kDa protein-like	180	71363	6/6
64	Heat shock protein 83	147	80785	6/6
47	Heat shock protein 81-1-like	165	80424	4/4
45	18.1 kDa class I heat shock protein-like	167	17472	3/3
46	18.1 kDa class I heat shock protein-like	166	17471	3/3
77	Heat shock protein 81-1-like	128	80164	3/3
80	Heat shock protein 83-like	125	90269	2/2
Functional category 7: *Transporters*/transport ATPases				
22	Importin subunit alpha-1b-like	254	59067	5/5
30	Importin subunit alpha-1b-like	212	59059	5/5
82	V-type proton ATPase subunit B 2-like isoform X1	124	54493	4/4
83	Importin subunit β-1-like isoform X1	124	97926	2/2
24	V-type proton ATPase catalytic subunit A-like	239	68800	8/8

25	ATPase alpha subunit, partial (mitochondrion)	224	49016	7/7
34	V-type proton ATPase catalytic subunit A	201	68575	7/7
93	V-type proton ATPase subunit C-like isoform X1	115	42390	3/3
Functional category 8: *Intracellular traffic/ mitochondrial*				
70	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial-like	138	70551	4/4
59	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial isoform X1	152	70559	3/3
81	Mitochondrial-processing peptidase subunit alpha-like	125	54958	3/3
Functional category 10: *Single transduction*/kinases/ phosphatases				
75	Phospholipase D alpha 1-like	131	92203	5/5
103	Phospholipase D alpha 1	104	93029	3/3
Functional category 11: Disease/defense/resistance genes/ defense-regulated / cell death / cell rescue / stress responses/detoxification /pathogenesis-related protein				
2	Catalase isozyme 2	968	57426	32/32
8	Catalase isozyme 2	461	56378	13/13
53	Catalase isozyme 1	159	57421	8/8
27	Aldehyde dehydrogenase family 2 member B7, mitochondrial-like	219	59169	7/7
Functional category 12: Unclear classification				
12	Chaperone protein ClpB1-like	374	101484	14/14
10	21 kDa protein-like	404	20682	13/13
14	Adenosylhomocysteinase-like	307	53784	8/8
15	Annexin D1-like	277	35912	8/8
20	4-coumarate--CoA ligase-like 10	269	55969	8/8
60	Glutamate decarboxylase 1-like	151	56240	6/6
21	Ultraviolet-B receptor UVR8 isoform X1	258	62622	5/5
29	Probable carboxylesterase 15	215	39388	5/5
48	Annexin D2-like isoform X1	163	35929	5/5
76	Selenium-binding protein 1	130	54937	5/5
51	Phosphoribosylamine-glycine ligase, chloroplastic	160	46991	4/4
54	Xyloglucan endotransglucosylase/hydrolase protein 22-like	158	31756	4/4
68	Elongation factor 2	140	95019	4/4
91	Alg-2-interacting protein x-like protein (Alix), also known as AIP. It is concentrated in phagosomes and exosomes and regulates apoptosis which means cell death.	117	96473	4/4
92	Cysteine desulfurase 1, mitochondrial-like	115	49654	4/4
43	Xyloglucan endotransglucosylase/hydrolase protein 2-like	169	33790	3/3

52	Agmatine deiminase isoform X1	160	43565	3/3
66	Extracellular ribonuclease LE-like	141	25859	3/3
85	Peroxisomal acyl-coenzyme A oxidase 1-like isoform X1	121	74557	3/3
87	UTP--glucose-1-phosphate uridylyltransferase	120	51903	3/3
88	Uncharacterized protein LOC103716547	120	49386	3/3
89	Pectinesterase	118	56836	3/3
99	Coronatine-insensitive protein 1-like	110	66744	3/3
109	Probable nucleoredoxin 1-1	101	66177	3/3
111	3-ketoacyl-CoA	100	48996	3/3
67	Peroxisome biogenesis protein 7	141	36000	2/2
100	Clavaminate synthase-like protein At3g21360]	108	36800	2/2

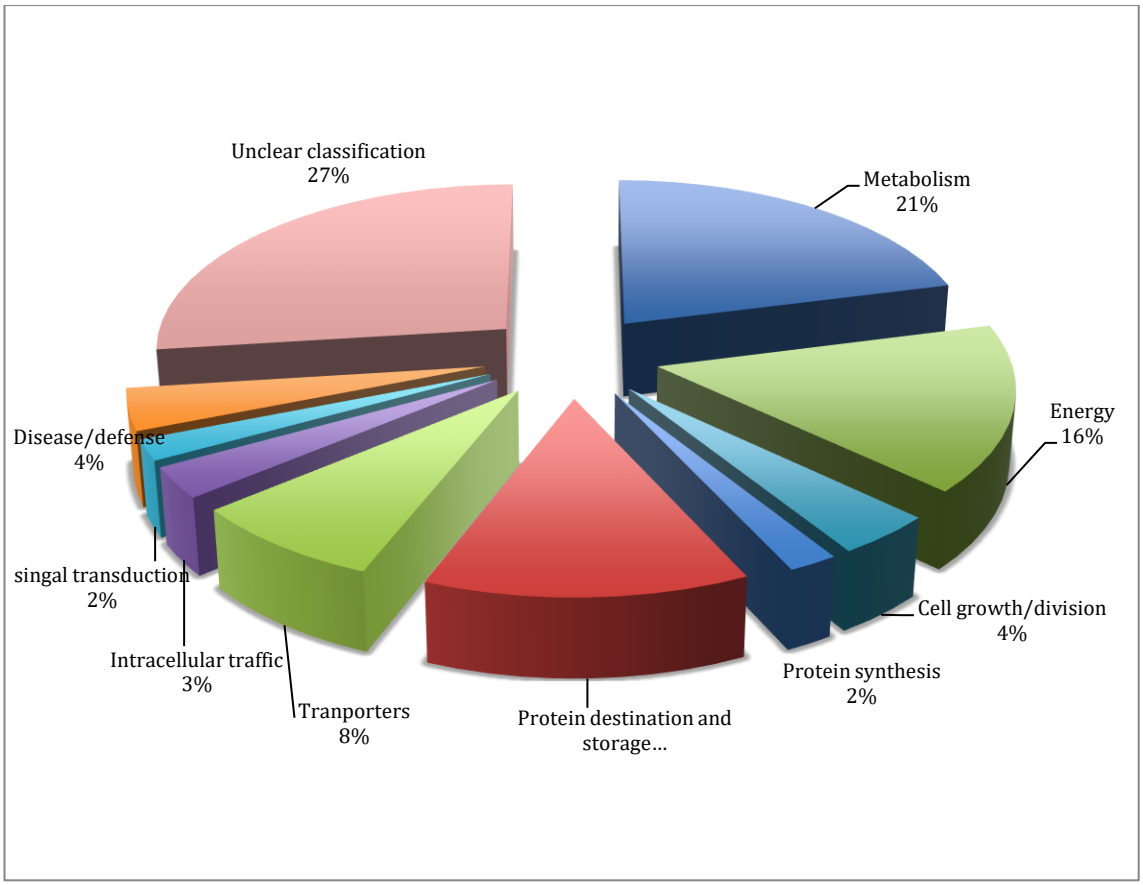


Figure 4.9: The percentage of the 99 proteins in DFPE identified by Liquid-chromatography Coupled Mass Spectrometry (LC-MS/MS) classified into 11 groups based on the functional categories according to Bevan et al., (1998).

The figure above shows that of the 100 selected proteins, 21% were related to metabolic function, 16% to energy function, and 13% to protein destination and storage function. The remaining 27% could not be classified. These results confirm the findings of (Al-

Khayri et al., 2017) who conducted proteomic analysis of an extract of date fruit protein and reported that the major protein fractions are related to metabolic function.

Table 4.8: The twenty-one proteins of DFPE with highest MOWSE score and lowest hit number

HN	Protein's name	MOWSE Score	MW (kDa)	Protein match
1	Sorbitol dehydrogenase-like	1089	39134	44/43
2	Catalase isozyme 2	968	57426	32/32
3	Glyceraldehyde-3-phosphate dehydrogenase 3, Cytosolic	510	37102	15/15
4	Heat shock cognate 70 kDa protein	501	71558	14/13
5	ATP synthase subunit β, mitochondrial-like	497	59650	11/11
6	Heat shock cognate 70 kDa protein	483	71476	14/12
7	Heat shock cognate 70 kDa protein	465	71535	12/11
8	Catalase isozyme 2-like	461	56378	13/13
9	21 kDa protein-like	404	20682	13/13
10	Adenosylhomocysteinase	387	53806	8/8
11	Chaperone protein ClpB1-like	374	101484	14/14
12	β-fructofuranosidase, insoluble isoenzyme 3-like, partial	329	65112	14/13
13	Adenosylhomocysteinase-like	307	53784	8/8
14	Annexin D1-like	277	35912	8/8
15	Phosphoenolpyruvate carboxylase	274	110548	11/11
16	Isoflavone reductase-like protein	274	33855	9/9
17	Phosphoenolpyruvate carboxylase, housekeeping isozyme	271	111016	10/10
18	Fructose-bisphosphate aldolase cytoplasmic isozyme-like	269	39047	7/7
19	4-coumarate--CoA ligase-like 10	269	55969	8/8
20	Ultraviolet-B receptor UVR8 isoform X1	258	62622	5/5

The highest MOWSE score protein in DFPE was identified by LC-MS/MS analysis as a sorbitol dehydrogenase-like protein with MW (kDa) 39134, which accounts for 16% of the proteins in the sample and falls in the category function of energy related proteins. This could correspond to the diffuse protein band range between 36 and 55 kDa seen in the SDS PAGE gel in **Figure 4.8**. The ultraviolet-B receptor UVR8 isoform X1 (62 kDa) could also be part of the diffuse range of bands.

The second highest MOWSE score protein was catalase isozyme 2 with MW (kDa) 57426, which accounts for 4% of the proteins in the sample and falls in the category

function of disease/defense proteins. This could correspond to the protein band of around 64 kDa in the SDS PAGE gel shown in **Figure 4.8**.

Other major protein bands in the SDS PAGE in **Figure 4.8** are 16, 100, and 149 kDa. The band at 16 kDa could correlate to 21 kDa protein-like with MW (kDa) 20682. The molecular weight of the respective proteins could have been increased due to contamination with fibres or starch. The band at 100 kDa could be the chaperone protein ClpB1-like with MW (kDa) 101484, while the band at 149 kDa could be the phosphoenolpyruvate carboxylase housekeeping isozyme with MW (kDa) 111016, which falls in the category function of energy-related proteins. A SCAR-like protein with a MW of 252 and 258 kDa identified in DFPE by LC-MS/MS difficult to detect by SDS-PAGE (**Figure 4.8, Lane 2, p.110**). However, there is a darkly stained diffuse area visible for DFPE in the region of 250 kDa, which could include a protein of this molecular weight. It was difficult to obtain clear peptide profiles for DPFE, despite attempts to optimize sample preparation, such as precipitation by TCA. The appearance of diffuse bands could be due to the presence of contaminating fibres or carbohydrates.

4.4.5 Structure and function of main proteins in DFPE

Sorbitol dehydrogenase

Sorbitol dehydrogenase (SDH) is a cytosolic enzyme which is present in several types of tissues, where it catalyzes the reversible oxidation-reduction of sorbitol, fructose, and nicotinamide adenine dinucleotide phosphate (Faqi, 2017). The metabolism of sorbitol also supports the plant's ability to withstand environmental stresses. In some fruits, such as sour cherry, the sorbitol levels dramatically increase during ripening, which can contribute to the sweetness of the fruit. There are two enzymes that have been specified in sorbitol conversion, which are NAD-dependent sorbitol dehydrogenase and sorbitol oxidase (Aguayo et al., 2013).

Catalase isozyme 2

Catalase is one of the major antioxidant enzymes present in all aerobic organisms: it converts hydrogen peroxide into water and oxygen gas and is thought to play a role in the protection of these organisms from the toxic effects of hydrogen peroxide (Inagi, 2011). Catalase plays a basic role in breaking down H_2O_2 accumulated in peroxisomes (Hu et al., 2010). Catalase is located in sites of H_2O_2 production in the cellular environment, such as mitochondria, peroxisomes, chloroplasts and cytosol of higher plants; it contains a haeme moiety at the active site and converts two hydrogen peroxide molecules to oxygen and H_2O (Ahmad, 2014). It can exist in multiple molecular forms or isozymes encoded by multiple genes, in any organism.

Phosphoenolpyruvate carboxylase

Phosphoenolpyruvate carboxylase (PEPC) is an enzyme from the carboxylase family which has been found in some bacteria (Chlorobiontes) and higher plants (Embryophytes) but not in fungi or animals (Kai et al., 2003). The significant role of PEPC is in assimilating atmospheric CO_2 through the Crassulacean acid metabolism photosynthesis (the CAM cycle). PEPC catalyzes the fixation of carbon dioxide with phosphoenolpyruvate to produce oxaloacetate, phosphate, GTP and GDP **Figure 4.10**.

It can also contribute in several non-photosynthetic processes, such as fruit ripening, seed formation, germination, and regulation of plants' tolerance to stresses and supporting carbon–nitrogen interactions (Wang et al., 2016).

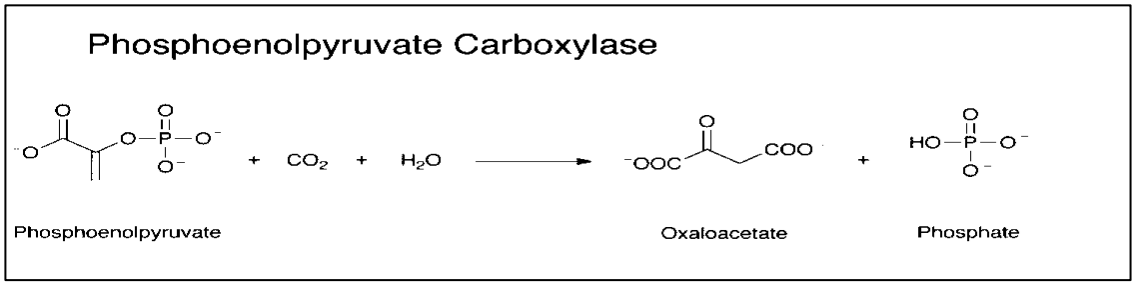


Figure 4.10: Enzymatic reaction of Phosphoenolpyruvate carboxylase (PEPC) (Worthington Enzyme Manual, 2019).

Ultraviolet-B receptor UVR8 isoform X1

Ultraviolet-B receptor, also known as UV-B resistance 8 (UVR8), is a UV-B – sensing protein common in plants (Christie et al., 2012). It senses ultraviolet light in the range of 280 to 315 nm, starting the response to plant stress. It is very sensitive at 285 nm, close to the lower limit of UVB.

21 kDa protein-like & chaperone protein ClpB1-like

Plants responded not only to climatic factors, but also biotic factors and soil factors. All these factors could put the plants under stresses and can excite some mechanisms of defense in plants. The response to stresses at the molecular level leads to an increase in the synthesis of heat-shock proteins (Hsps) (Al-Whaibi, 2011). The 21 kDa protein in DFPE sample is one of the small heat-shock proteins (sHsps). Hsps could protect cells from the effect of denaturation (Neta-Sharir et al, 2005).

4.5 Conclusions

The first aim of the research reported in this chapter was to extract the proteins from date palm fruit at the *Tamr* stage of ripening. Different methods of protein extraction were applied to date fruit. A protein extract containing 25.8% protein per dry weight was obtained, which is a 13-fold enrichment compared to the 2.8% in the initial sample (before extraction). This is the highest content of protein extracted from date fruit reported for the first time. The protein yield was 51% indicating that the extraction process could be improved to increase the yield.

Different methods of extraction were tried. A significant problem affecting the protein yield was the effect of proteases, evidenced by the improved yield (87% yield) when extraction was carried out in the presence of protease inhibitors. As protease inhibitors are not acceptable for use in food processing, thermal treatment at neutral pH was applied followed by acid precipitation. Resuspension of the resulting pellet in water, adjusting to

neutral pH and repeating the acid precipitation step resulted in a protein extract of 25.8% protein at 51% yield. The final process used for preparation of the protein extract is **Method 1.1**, depicted in **Figure 4.6**. The extraction process resulted in 4.2% DFPE, 57.1% date syrup and 38.6% waste. DFPE contains 49% protein whereas the rest was in the date syrup 26.7% and 24.3% in the waste. The protein concentration in the waste was calculated and not measured, because we focused on the measurement of the concentration of protein in the protein extract and tried to solve the loss of protein yield due to proteases.

The development of a protein extraction process suitable to the food industry, involving heat treatment and repeated steps of acid precipitation (HCl) and re-solubilisation of protein with NaOH is reported. Further optimization of the process would be possible by optimization of a) the pH for solubilization of the protein and subsequent acid precipitation and b) temperature and time of thermal treatment to inhibit proteases to achieve higher protein yields.

Acid precipitation (iso-electric precipitation) of proteins is known to the food industry for extraction of soy protein isolate and pea protein (Stonea et al., 2015), and the application of thermal treatment to improve yield of black-eyed bean proteins have been reported by Campbell et al., (2016). The novelty of the current process is application of the thermal denaturation of the date proteins, which increased the yield when precipitated by acid, as well as inhibited protease activity.

The second aim was to examine the electrophoretic profile of DFPE. It was difficult to obtain clear electrophoretic bands of DFPE, despite repeated attempts to purify the protein further for electrophoresis. This could have been caused by contaminating fibres, or the diffuse bands could be a result of proteolysis.

The third aim was to characterise the proteins by LC-MS/MS and to match the results to an existing database. Five hundred and fifteen proteins were identified by LC-MS/MS

and out of those 100 were selected based on MOWSE score and HN number. The major proteins of DFPE were related to the metabolic function, which accounts for 21%, while 16% of the sample protein is related to the energy function, and 13% to protein destination and storage function. The remaining 27% could not be classified. The selection was further narrowed down to 21 proteins with the highest MOWSE score (**Table 4.8)**. The two most abundant proteins with the highest MOWSE score were identified as sorbitol dehydrogenase-like with MW (kDa) 39, which is an energy protein which represents 16% of the proteins in DFPE. Catalase isozyme 2 with MW (kDa) 57, which protects the cells form oxidative damage, accounted for 4% of the proteins in DFPE. The protein bands of corresponding molecular weight were identified by SDS PAGE analysis. These results are novel in so far as the proteins in a food grade protein extract are characterized compared to Al-Khayri et al., (2017) who conducted proteomic analysis on a date fruit protein that were extracted using toxic chemicals which are phenylmethylsulfonyl fluoride (PMSF) and mercaptoethanol and therefore not comparable to methods used in the present study which aims at developing an extraction method suitable for use in the food industry. The authors published a photograph of an SDS-PAGE gel of the protein extract, but the sizes of the molecular weight markers were not indicated neither was the electrophoretic profile discussed.

CHAPTER FIVE

Development of an infant cereal product with reduced allergenicity

5.1 Introduction

There are many of children under the age of five are allergic to milk, eggs, soy or wheat; most common causes allergy in an infant are milk and egg (Mehta et al., 2013). In many countries, infant cereals are the first solid food that is introduced to infants (Klerks et al., 2019) because they provide energy, carbohydrates, starch, fibre, proteins, vitamins and minerals as exclusive breastfeeding is no longer sufficient to meet the nutritional requirements for an infant over 6 months. They are also a good source of iron when the infant's iron stores are depleting (Fardet, 2010; Domellöf et al., 2014). Cereals also provide non-digestible carbohydrates, which lead to the development of microbiota in the gut (Fallani et al., 2011). Cereals have a semi-solid texture and consistency, it also has a mild taste, which lead infants to accept solid foods at the beginning of complementary feeding (Sakashita et al., 2013). In the past, the standard recommendation was avoiding giving cereals to infants to prevent introduction of allergenic food components to infants. However, today there is a recommendation by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition to introduce iron-rich complementary foods, such as cereals from four months alongside breastfeeding, while the European Food Safety Authority recommended to offer iron-rich food for infant after four to six months (Domellöf et al., 2014; Fewtrell et al., 2017). However, in New Zealand and the United States, there is a recommendation to avoid introduction of cereals under the age of six months (Klerks et al., 2019), which is attributed to the outer layers of the cereal grain likely to have been exposed to contaminants, such as mycotoxins and heavy metals (Thielecke and Nugent, 2018).

Infant cereal products on the market

So far, offering cereals with or without gluten for infants under six months of age is a general issue and there is no agreement has been reached about it, while Spain and France recommended not offering cereals with gluten to infants under six months (Dalmau et al., 2017). Cereals provided to infants could be affected by the cultural beliefs of the countries cultural, while in some countries, it is popular to provide an infant with cereals contains gluten. For instance, in African countries the first solid food for infants is maize porridges in the UK and Ireland the most consumed infant cereal is rice-based, while in Spain the most popular first complementary food is wheat or rice cereal (Tarrant et al., 2010; O'Donovan et al., 2015; Klerks et al., 2019). Manufacturers that produce commercial infant food must follow the regulations relating to the age of introduction of solids to bring them into line with global infant feeding guidance (Crawley and Westland, 2017). We have chosen (Nestlé) cereal as a control sample because it is the most common infant food in the KSA. Most of the commercial infant cereals in the market in Arab countries, especially in the KSA are imported from EU countries or the United States in limited brands, which are expensive. Lack of availability forces them to order infant cereal using internet websites, which are costly and have up to 2 weeks delivery time.

5.1.1 Principles of methods used in this chapter

5.1.1.1 Cereal recipe design and water activity during shelf life

The recipe was designed to obtain an adequate nutritional composition for an infant under 5 years of age. The nutritional composition of CERELAC (Nestlé) formula for an infant less than 5 years of age was used as control. Milk protein was replaced by date fruit protein extract and wheat flour was replaced by brown rice flour to render it gluten-free. Date fruit protein extract (syrup) prepared as a by-product from protein extraction **Chapter 4**, **Section 4.3.3** and molasses were used as sweeteners instead of sugar. The nutritional composition of the DFPE cereal was compared to that of a commercially

available cereal formulation for infant under 5 years of age (CERELAC) and the nutritional composition was compared to that of the Reference Nutrient Intake (RNI) for infants of that age group.

The approach followed in the present study was to gain information available in the literature of the chemical composition of each ingredient to be used in the date cereal recipe. Using this information, a recipe was formulated in Excel to calculate the contribution of moisture, fat, protein, carbohydrate and fibre of each ingredient, based on information of the nutritional requirements for children at the age between (13-36 months) obtained from literature (NCBI, 1968).

Water activity (aw)

Water activity (a_w) or available water is one of the most significant factors used to determine the storage stability y of a food product. Water activity helps the food designers to predict the microbiological stability of a food and formulate shelf life of food products and it has an important effect on the colour, flavour and aroma of the food. It can be defined as the ratio of water vapour pressure of food substrate to that of vapour pressure of pure water at the same temperature (Tucker, 2015)

$a_w = p/p_o$

where **p** is the vapour pressure of the solution and $\mathbf{p_o}$ is the vapour pressure of the solvent. Water activity in foods depends on the kind of solutes and their concentrations also it affects the physical properties of food for instance, vapour pressure (water activity), density, boiling point and freezing point (Ergun et al., 2010). Water activity plays a significant function in various processes, such as spontaneous autocatalytic lipid oxidation reactions, reducing non-enzymatic browning reactions and affecting the activity of vitamins and enzymes (Sandulachi, 2012). The value for a_w ranges from (0) absence of water to (1.0) pure water. Most of spoilage bacteria require at least a water activity 0.90 to grow and most of spoilage yeasts require at least a water activity 0.88 to grow; in

the range of water activity less than 0.65 there occurs no microbial growth (Erkmen and Bozoğlu, 2016). Water activity is sometimes described as the amounts of free and bound water available in food product. The measure of the tightness of "bound" water and the relationship to the work needed for water removal from the system is known as water activity (Nelson, 1995).

5.1.1.2 Porridge design and sensory testing

To make a porridge with DFPE, it was dissolved in camel milk to compensate for some deficient amino acids in DFPE and to increase the calcium content. Camel milk is widely consumed in the UAE and Northern Africa. Recently, scientists demonstrated its superiority to cow's milk for patients who suffering from lactose intolerance or milk allergies because it does not contain (β-lactoglobulin) which is the protein that causes allergic reactions to cow's milk (Shabo et al., 2005). To make a porridge with CERELAC, it was dissolved in cow's milk. The nutritional composition of the two porridges was compared to the RNI for infants of that age group. The porridges were subjected to sensory analysis.

Sensory evaluation

Sensory evaluation or sensory analysis refers to a scientific discipline that applies basics of statistical analysis and experimental design to the use of human senses (smell, sight, taste, hearing and touch) for the objective testing of food products. Sensory description of food is different from chemical analysis of food. The language used in the sensory description is more global, less accurate and uses published definitions of sensory attributes (Sune et al., 2002).

5.2 Materials and methods

5.2.1 Materials

DFPE was used that was prepared as described in **Chapter 4**, **Section 4.3.1**, **Method 1.1**. Date fruit syrup **Figure 5.1** as prepared on **Chapter 4**, **Section 4.3.3**. Brown rice flour (gluten-free) obtained from Holland and Barrett, Edinburgh. Vegetable oil, meridian blackstrap molasses and cow's milk powder were obtained from Tesco, Edinburgh. Camel milk powder was obtained from (Desert Farms, Amazon.co.uk). The cereal used as control was CERELAC (Nestlé) purchased from Maqbool supermarket, Edinburgh.

Figure 5.1: Date fruit syrup.

5.2.2 Methods

5.2.2.1 Recipe design and nutritional evaluation

The recipe was designed using chemical and nutritional results of DFPE and date syrup using results of chemical composition analysis, amino acids analysis and mineral analysis as described in **(Chapter 2**, **Figure 2.6**, **2.8** and **2.10)** of the thesis. The cereal was prepared by grinding a mixture of DFPE powder, brown rice flour, date syrup, vegetable oil and molasses using (VonShef coffee grinder -200W 2 in 1 stainless steel blade) for 2

min. The chemical composition, amino acid and mineral content of brown rice flour, vegetable oil and molasses were obtained from the literature. Using this information as well as the relevant information of DFPE in **Chapter 2**, a cereal recipe was formulated using Microsoft Excel. The amino acid composition of the finished product was analysed by (ALS laboratories, Chatteris, UK). Due to the limitations of time and the amount of date cereal sample, vitamin analysis of DFPE or date syrup was not carried out, neither of the finished product.

5.2.2.2 Water activity (a_w)

Water activity of the sample was measured at the starting time of storage and after 7 months storage at 37°C using a water activity meter (Novasina Labmaster), the measurements were made with triplicate samples.

5.2.2.3 Method for preparing porridge for sensory analysis

The date cereal sample and the CERELAC sample were mixed with camel and cow's milk (30°C), respectively, in quantities to obtain equal concentrations of protein in the samples. In date porridge, the powder of camel milk was dissolved in warm water (30°C) to prepare the date porridge, while cow's milk (30°C) was used to prepare the control porridge. (see **Table 5.8**).

The reason for choosing camel milk for preparing DFPE porridge was to increase the content of calcium and some amino acids (Lys, Met, Cys, Phe, Try and Tyr), which are in lower quantities in DFPE than the RNI.

5.2.2.4 Calculation of reference nutrient intake (RNI) of amino acids and minerals

Based on the World Health Organisation (2007), RNI of amino acid for children under the age of 5 should be calculate as (mg/average body weight) and the average body weight for the target group of the study is (10 kg) (see **Table 5.9** and **5.10**).

5.2.2.5 Sensory evaluation

Sensory evaluation (colour, aroma, the overall flavour, sweetness, bitterness, texture, and overall acceptability of the product). A sensory evaluation of the fresh date cereal sample was carried out according to Lustre et al., (2007) to investigate the overall of acceptability of the sample. Ten participants (who do not smoke, all participants were aged between 28 to 55 years; a higher proportion of females (6) than males (4). The participants were invited to participate from the School of Engineering and Physical Sciences (EPS), Heriot-Watt University. The evaluation of the cereal was based on an nine-point hedonic scale, with 1= disliked (extremely), 2=disliked (very much), 3= disliked (moderately), 4= disliked (slightly), 5= neither liked nor disliked, 6= liked (slightly), 7= liked (moderately), 8=liked (very much) and 9= (liked) extremely (Lustre et al., 2007).

5.3Results and discussions

5.3.1 Recipe design

Table 5.1: Percentage of ingredients in CERELAC commercial skim milk- based infant cereal.

Ingredients	Percentage (%)
Hydrolysed wheat flour	60.6
Skimmed milk powder	25.2
Vegetable oil	8.7
Honey	5.5
Total	**100**

Table 5.2: Percentage of ingredients in the date cereal recipe

Ingredients	Percentage (%)
Date protein extract (powder)	69
Brown rice flour (gluten-free)	19
Date syrup	5
Vegetable oil	4
Molasses	3
Total	**100**

Figure 5.2: A sample of final product (date cereal).

5.3.2 Nutritional composition of the cereal products and comparison to reference nutrient intake (RNI)

Table 5.3: Composition of the date cereal product

***Rec**: Recipe representing percentage of ingredients in the total recipe; **RM**: composition of each ingredient; **Prod**: contribution of sub-compounds of each ingredient to the final product recipe; **Carb**: carbohydrates.*

% Ingredient	%Rec	% Moisture		% Fat		% Protein		% Carb		%Fibre	
		RM	Prod	RM	Prod	RM	Prod	RM	Prod	RM	Prod
DFPE	69	15	10.0	0.3	0.2	25	17.0	43	29.0	3	2.0
Rice flour	19	11	2.0	0.7	0.1	7	1.0	79	15.0	0.2	0
Date syrup	5	19	1.0	0.3	0	1	0.1	73	3.0	1	0.1
Oil	4	0	0	100	4.0	0	0	0	0	0	0
Molasses	3	21	0.7	0	0	0	0	74	2.0	0	0
Total	**100**		**14**		**4**		**19**		**51**		**2**

Table 5.4 compares the compositions of date cereal sample with the control.

Contents	Date cereal (%)	CERELAC cereal (%)
Carbohydrates	51	68
Protein	19	15
Fibre	2	2
Fat	4	10

The date cereal sample (100 g), which is in powder condition before mixing it with the liquid of camel milk, has 51% carbohydrates, 2% fibre, 4% fat and 19% protein. The protein content of date cereal is higher than the CERELAC cereal which is a (100 g) of

control sample before mixing it with the liquid of cow's milk. The date cereal has only 4% fat, that is lower than the CERELAC cereal sample (10%).

Table 5.4: Essential amino acids composition of date cereal compared to the CERELAC cereal.

Amino acid	CERELAC cereal (mg/100 g)	Date cereal (mg/100 g)
Histidine	356	240
Isoleucine	749	400
Leucine	1273	650
Lysine	890	430
Methionine	376	180
Cysteine	132	130
Phenylalanine	674	460
Tryptophan	187	15
Valin	912	550
Tyrosine	668	340
Threonine	562	460
Total	**6784**	**3855**

The content of essential amino acids in date cereal in **Tables 5.5** are approximately 50% lower than that in CERELAC cereal.

Table 5.5: Mineral content of the date cereal compared to CERELAC cereal

	Calcium (mg/100 g)	Iron (mg/100 g)	Zinc (mg/100 g)
Date cereal	8	19	2
CERELAC cereal	400	9	3

As can see from **Table 5.6**, the content of calcium and zinc in 100g of date cereal are lower than CERELAC cereal (8 mg and 2 mg versus 400 mg and 3 mg). While the content of iron in date cereal is more than CERELAC cereal (19 mg versus 9 mg) in CERELAC cereal. The result indicates that, date cereal could be good source of iron for infant.

The results indicated in **Table 5.4**, **5.5** and **5.6** compare the nutritional composition of date cereal to that of CERELAC cereal. These results show that, date cereal contains less carbohydrate, fat, calcium, zinc and less all amino acids, while it has a higher content of iron than CERELAC cereal.

The results show that the date cereal has about 50% less essential amino acids than the CERELAC product. In order to match the amino acid nutrition of CEREAL, double the portion of date cereal has to be consumed or alternatively that date cereal could be made up with camel milk to improve the nutritional value (see next section). The calcium content could be improved by supplementation with calcium. For instance, calcium carbonate could be added that is an approved additive for nutritional purposes (EFSA Directive 2002/46/EC relating to food additives). The fat could be supplemented by other food products in the diet.

5.3.3 Nutritional composition of porridges made with camel and cow's milk

In preparation for sensory evaluation, the date cereal was mixed with camel milk to make a porridge. The control (CERELAC) was mixed with cow's milk in a ratio to obtain an equal protein concentration to the date cereal porridge. **Table 5.8** presents the nutritional composition of ingredients in both porridges.

Table 5.6: Proximate composition of date and control porridge samples. [a] is the content of lactose in camel and cow's milk. The abbreviations used in this table are explained in the legend of **Table 5.3.**

% Ingredient	**%Rec**	**% Moisture**		**% Fat**		**% Protein**		**% Carb**		**% Fibre**	
		RM	Prod	RM	Prod	RM	Prod	RM	Prod	RM	Prod
Date cereal	35	14	4.9	4	1.4	19	6.6	51	17.8	2	0.7
Camel milk powder	15	1	0.1	4	0.6	3.6	0.5	4.2	0.6[a]	0	0
Water	50	100	50	0	0	0	0	0	0	0	0
Total	**100**	**0**	**55**	**100**	**2**	**0**	**7.1**	**0**	**18.4**	**0**	**0.7**
CERELAC cereal	44	9.1	4	10	4.4	15	6.6	68	29.9	2	0.8
Cow's milk powder	16	2.5	0.4	3.8	0.6	3.2	0.5	5.3	0.8[a]	0	0
Water	40	100	40	0	0		0		0		0
Total	**100**		**44.4**		**5.0**		**7.1**		**30.7**		**0.8**

Table 5.7: Comparison of Reference Nutrient Intake (RNI) for infants compared to the composition of the date and CERELAC porridges. The information for RNI is obtained from (NCBI, 1968).

	Carb	**Total fibre**	**Fat**	**Protein**	**Calories**
RNI for 13-36 months (g/day)	130	19	33-44	19	1197
Date porridge (g/100 g)	18.4	0.7	2	7.1	27.5
CERELAC porridge (g/100 g)	30.7	0.8	5	7.1	42.8

The recommended daily consumption of the CERELAC porridge for infants is up to 2 portions per day (one portion is 50 g). **Table 5.8** shows the percentage of RNI of proximate components of 2 portions of each type of cereal porridges. The table shows that 100 g of CERELAC porridge, which is 2 portions, offers less carbohydrate than date porridge, but similar quantities of fat, fibre and protein. The lower carbohydrate of date porridge results in almost half the calories than CERELAC porridge. The RNI of fat for infants ranges from 30 to 40% of their total calories, which is equal (33-44 g/day) as 1 g of fat provides 9 kilocalories (nal.usda.gov, 2019).

These results indicate that double the quantity of date porridge is required to match the calorie intake of CERELAC porridge. The calorie intake could be compensated by consumption of other foods.

Table 5.8: Amino acid content of date and CERELAC porridges compared with RNI for 12-24 months (mg per average body weight in the table).

Amino acid	RNI for 12-24 months per 10 kg weight	Date porridge (mg/100 g)	CERELAC porridge (mg/100 g)	% RNI offered by date porridge	% RNI offered by CERELAC porridge
Histidine	190	519	380	273	200
Isoleucine	280	875	1497	312	534
Leucine	660	362	3600	54	545
Lysine	580	1140	1687	196	290
Methionine	250	618	1429	247	571
Cysteine	250	270	202	108	80
Phenylalanine	630	551	472	87	74
Tryptophan	110	275	306	250	278
Valin	350	912	1697	260	484
Tyrosine	630	164	342	26	54
Threonine	340	956	679	281	199

Table 5.9 shows that both porridges supply more than the RNI of most essential amino acids. Date cereal matches RNI of tyrosine and phenylalanine supplied by CERELAC porridge. However, date porridge is deficient in leucine compared to CERELAC which offers only 10% of RNI. This amino acid could be supplemented in the date cereal formulation.

Table 5.9: Mineral content of date porridge compared with RNI for 12-24 months

Minerals	RNI for 12-24 months (mg/day)	Date porridge (mg/100 g)	CERELAC porridge (mg/100 g)
Calcium	700	31.3	193.6
Zinc	3	0.79	1.36
Iron	7	6.67	3.91

Table 5.10 shows that the content of calcium in the date porridge compared to CERELAC, which is (4.4% of RNI versus 27.6% RNI) supplied by CERELAC. However, the zinc and iron in date porridge was 26.3% and 95.2% respectively of RNI, versus 45.3% and 55.8% of RNI in CERELAC porridge. The content of zinc in date porridge is less than CERELAC porridge while the content of iron in date porridge is higher.

Table 5.10: The content of anti-nutritional factors in 100 g of date porridge derived from DFPE and date syrup.

Anti-nutritional factors	(mg/100 g)
Oxalate	1.46
Tannic acid	0.05
Phytate	0.07

The results in **Table 5.11** show that, the content of oxalate, tannic acid and phytate in date porridge are 1.46, 0.05 and 0.07 mg/100 g wet weight porridge respectively. These values are far below the acceptable daily RNI of oxalate (for adults) of (44-352 mg/day); tannin 5 mg/100 g of food and phytate requirement of less than 10-60 mg/day (Elinge et al., 2012). It could be assumed that the date cereal porridge is safe to be consumed by infants in this regard.

Sensory evaluation

Figure 5.3 presents the sensory evaluation of the fresh date porridge given by 10 participants (details of participants are given in **Section 5.2.2.5)** compared with the CERELAC porridge. The participants were asked to evaluate the different attributes on a scale of 1 to 9, from disliked extremely (1) to liked extremely (9). The sensory attributes included colour, aroma, the overall flavour, sweetness, bitterness, texture, and overall acceptability of the product (Lustre et al., 2007). The results show that the average of the overall acceptability was 5.4 of 9. The average for the evaluation of the colour and aroma was 5.9 of 9 and for the overall flavor it was 6.1 of 9. The average of bitterness and texture for the date porridge sample were significantly lower than the CERELAC. Porridge. For bitterness the value was 5.7 of 9, while for texture it was 4.6 of 9.

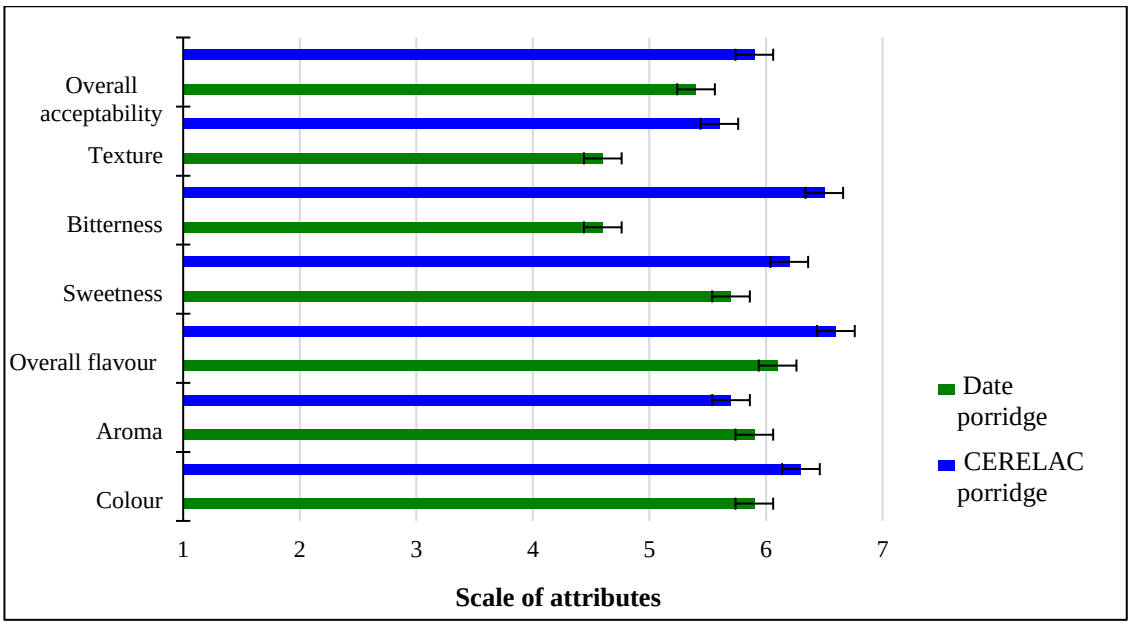


Figure 5.3: Sensory evaluation for date porridge compared with the CERELAC porridge. *1=disliked extremely, 2=disliked very much, 3=disliked moderately, 4=disliked slightly 5=neither liked nor disliked, 6=liked slightly, 7=liked moderately, 8=liked very much and 9=liked extremely.*

Table 5.11: The average of sensory evaluation for date porridge compared with the CERELAC porridge.

Attributes	Date porridge	CERELAC porridge	Significantly different?	Variance	p-value
Colour	5.9	6.3	No	0.08	0.02
Aroma	5.9	5.7	No	0.02	0.01
Overall flavour	6.1	6.6	No	0.12	0.02
Sweetness	5.7	6.2	No	0.12	0.02
Bitterness	4.6	6.5	Yes	1.80	0.10
Texture	4.6	5.6	Yes	0.50	0.06
Overall acceptability	5.4	5.9	No	0.12	0.02

All sensory attributes were rated similar to the CERELAC apart from bitterness and texture. The average value for bitterness and texture for date porridge was 4.6 of 9 which is lower than the CERELAC porridge 6.5 and 5.6 of 9, respectively. The texture of the date porridge was not as smooth as CERELAC (significantly different) which could be improved by grinding of the date porridge. The most concerning factor was that the date porridge was perceived as significantly more bitter than CERELAC, although it was still

within acceptable range (separate conversation by sensory panellists). The bitterness could be due to the presence of oxalate in the DFPE sample. Oxalate is responsible for the characteristic taste of spinach and rhubarb (Horie and Ito, 2006). Tannic acid is also responsible for bitterness taste. McGee and Harold (2004) reported that consumption of unripen fruit or having red wine or tea led to the feeling of dry mouth due to the presence of tannins in these foods.

5.3.4 Water activity (a_w)

Figure 5.4 displays result of water activity of the date cereal and CERELAC samples which were measured freshly and after 7 months storage at 37°C. The results indicate that there was no significant increase in a_w during storage for both samples indicating that the date cereal could be stable with respect to microbial growth during storage.

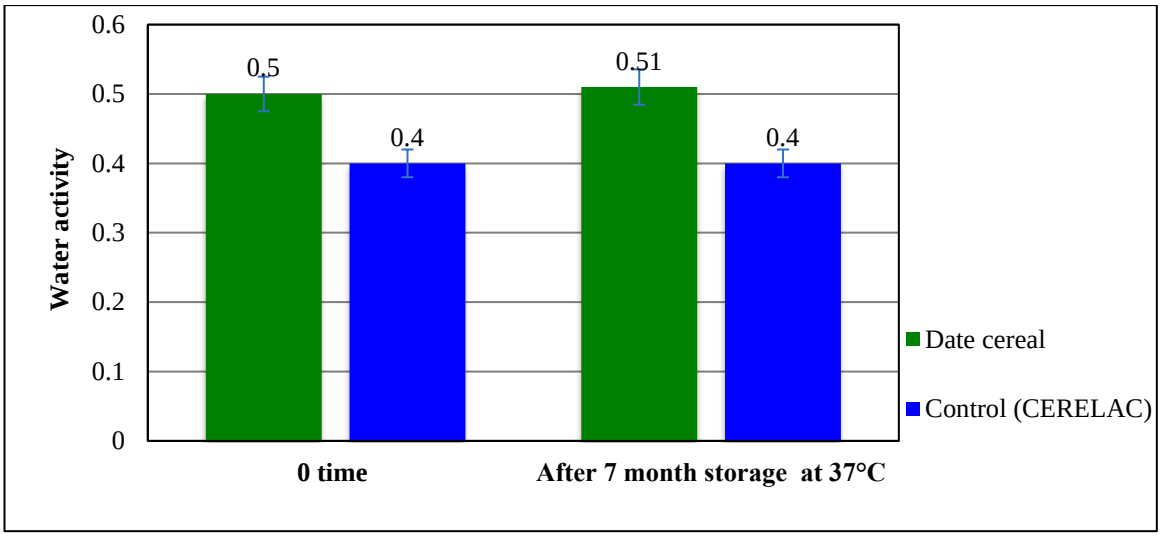


Figure 5.4: Water activity of the date cereal at zero time and after seven months storage at 37°C compared with the control sample.

5.5 Conclusions

The date cereal formulation requires further improvement to be potentially marketed as an infant food and competitor to CERELAC. It would need to be supplemented with calcium and leucine essential amino acid. Furthermore, instructions should be given on the packaging to make the porridge with camel milk. If these instructions are followed, the 100 g portion of date cereal porridge would provide essential amino acids and minerals similar to CERELAC porridge, but with lower calorific value.

A date cereal porridge prepared with camel milk had a protein, fibre and zinc composition similar to that of a porridge made with CERELAC prepared with cow's milk. However, it contained less carbohydrates, fat, calcium and had a lower calorific value. Date cereal porridge contained significantly more iron than CERELAC porridge, which is a desirable nutritional aspect for infants as discussed in the introduction.

A concerning factor was that the date cereal porridge was perceived as significantly more bitter than the control porridge, although it was still within acceptable range (separate conversation by sensory panellists). This could be ascribed to the content of the anti-nutritional factors oxalate and tannin, which have a bitter taste. However, the concentration of these anti-nutritional factors was far below the acceptable daily intake for adults and it is assumed that it would not be of concern in infant cereal.

The date cereal product made with camel milk would offer the advantage to CERELAC porridge that it is gluten-free and free of cow's milk, thereby it could be labelled as less allergenic than CERELAC porridge. Camel milk is widely consumed in the KSA, UAE and Northern Africa. Scientists demonstrated its superiority to cow's milk for patients who suffering from lactose intolerance (Desert farms, 2015; Cordoso et al., 2010). Camel milk is also less allergenic because it includes a different β-casein to cow's milk and it does not contain (β-lactoglobulin and β-casein) which are the proteins that causes allergic

reactions to cow's milk (Shabo et al., 2005). Camel's milk also contains a some of protective proteins, such as peptidoglycan recognition protein, lysozyme, lactoperoxidase and lactoferrin, these proteins could have a role for enhancing immune defence mechanism (Abrhaley and Leta, 2018). The immunoglobulins in camel's milk are similar to that in mothers' milk, that compound reduces the allergic reactions in children and strengthens their future response to foods (Abrhaley and Leta, 2018). Restani et al., (1999) indicated that, drinking camel milk by children with food allergies has a positive, rapid and long-lasting impact. Based on findings by Zibaee et al., (2015), camel milk is safe to be consumed by children suffering from or at risk of developing cow's milk allergy. The date porridge contains 60% plant protein whereas all protein in CERELAC porridge is derived from cow's milk. The sweeteners in date cereal are date syrup and molasses of which contain natural sugars 61.7 and 56%, respectively. The date cereal also offers excellent anti-oxidant properties similar to vitamin C. As cow's milk does not contain anti-oxidants, CERELAC is supplemented with vitamin C. Date cereal must not be used as a substitute for a varied and balanced diet for infants; it can be used as a supplement besides to having a varied diet. According to the recommendation of NHS (2018), young children should eat a variety of fruits, vegetables, meat, fish, egg, nuts, beans, and starchy foods to get a balanced diet.

CHAPTER SIX

Conclusions and recommendations

The novel results reported in this PhD study are as follows:

1. Proximate composition, PDCAAS value, anti-oxidant and anti-nutritional properties of the extract (**Chapter 2**).
2. Determination of physicochemical and functional properties of the protein extract and the respective effect of thermal treatment (**Chapter 3**).
3. Development of an extraction process of protein from date fruit that is suitable for adaption/implementation in the food industry; resulting in a protein extract. with higher protein concentration than reported before (**Chapter 4**).
4. SDS PAGE and proteomic analysis of the protein extract (**Chapter 4**).
5. Development of a plant-based infant cereal product with potential reduced allergenicity (**Chapter 5**).

These points are discussed in detail below:

1. **Proximate composition, PDCAAS value, anti-oxidant and anti-nutritional properties of the extract**

Proximate composition**,** mineral analysis, anti-oxidant, amino acid composition and digestibility analysis of DFPE showed a relatively high protein content reported for the first time, with all essential amino acids, high source of iron and with excellent anti-oxidant properties matching that of ascorbic acid. The extract had a lower PDCAAS value than SPI and contained anti-nutritional factors oxalate, tannin and phytate, yet at low quantities that is assumed not to be of anti-nutritional concern. This leads to the conclusion that DFPE could not fully replace SPI on a nutritional basis. On the other hand, DFPE could be used in combination with other plant protein extracts currently emerging, such as pea protein isolate in food applications to replace soy protein. Furthermore, the PDCAAS value could be improved by improving the extraction process to provide a purer protein extract with higher concentration.

2. Physicochemical and functional properties of the extract and the respective effect of thermal treatment

The concentration of free and total sulphydryl groups were significantly less that for SPI, confirming the results of low cysteine of amino acid analysis results of DFPE (**Chapter 2**). The results indicate that DFPE is not as thermally stable as SPI, whilst considering the fact that DFPE had been subjected to heat treatment during the extraction process. The effect of thermal treatment on SH groups profile indicate that DFPE is not as thermally stable as SPI, whilst considering the fact that DFPE had been subjected to heat treatment during the extraction process. This physicochemical profile was mirrored by the corresponding decrease in functionality including decrease at longer heating times in solubility, foam capacity emulsion stability index and increased water separation in emulsions.

The excellent solubility of DFPE could enable its application in baby food, beverages and drinks such as smoothies with a fairly neutral pH, to enhance the protein content and replace soy protein. Foaming properties indicate that DFPE could be used in foamed products provided that it is not exposed to excessive heat treatment. The DFPE powder showed water absorption capacity and oil absorption capacity in the range required for application in soups, cake batters and sausages and nutrition bars. On the other hand, it is evident that DFPE in solution is not as good an emulsifier as SPI and therefore will not be able to replace SPI in oil in water emulsions where the protein requires to be soluble in the water phase. This applies to products, such as mayonnaise and pasteurised fat-containing sauces.

Further studies are required to obtain an optimal balance between thermal treatment of date protein during the extraction process and the heat stability of the ingredient to withstand pasteurisation temperatures during food processing to maintaining its functional properties.

3. Development of an extraction process to produce a protein concentrate

A protein extract containing 25.8% protein per dry weight was obtained which is a 13-fold enrichment compared to the 2.8% in the initial sample (before extraction). This is the highest content of protein extracted from date fruit reported for the first time. The protein yield was 51% indicating that the extraction process could be improved to increase the yield. A significant problem affecting the yield was the effect of proteases, evidenced by the improved yield when extracted in the presence of protease inhibitors. As protease inhibitors are not acceptable for use in food processing, the process requires further optimization by variation in pH, temperature and duration of treatment to inhibit proteases to achieve higher protein yields.

The development of a protein extraction process suitable to the food industry, involving heat treatment and repeated steps of acid precipitation (20% acetic acid) and re-solubilisation of protein with NaOH. The novelty of the current process is the thermal denaturation of the proteins, which increased the yield when precipitated by acid, as well as inhibited proteases activity. Further optimisation of the process is required to 1) inhibit proteases in order to improve the yield and 2) to optimise protein functionality as discussed in **Chapter 3**. In-line control of protein denaturation during thermal processing would be required to achieve the optimal balance between inactivation of proteases whilst maintaining protein functionality. Such monitoring technology has been patented by Campbell (2007) and is applied to whey protein in the food industry but still has to be exploited for plant proteins.

The extraction process resulted in 4.2% DFPE, 57.2% date syrup and 38.6% waste. DFPE contains 49% protein whereas the rest was in the date syrup 26.7% and 24.3% in the waste (which could also be lost due to protease activity). A date syrup which contained most of the sugar and carbohydrate was developed which showed that the by-product could be utilized and is also potentially marketable as a food product. The

waste (containing mostly fibre) might be used as animal feed. Further investigations could lead to identification of the fibre and evaluation of the properties as food ingredient.

4. SDS PAGE and proteomic analysis

The two most abundant proteins with the highest MOWSE score were identified as sorbitol dehydrogenase-like with MW (kDa) 39, which is an energy protein which represents 16% of the proteins in DFPE. Catalase isozyme 2 with MW (kDa) 57, which is disease/defense related, accounted for 4% of the proteins in DFPE. The protein bands of corresponding molecular weight were identified by SDS PAGE analysis. These results are novel in so far as the proteins in a food grade protein extract are characterized compared to Al-Khayri et al., (2017) who conducted proteomic analysis on a date fruit protein that were extracted using toxic chemicals.

5. Development of a plant-based infant cereal product with potential reduced allergenicity

An infant cereal based on date fruit protein was developed as a potential competitor to a commercial infant cereal (CERELAC) sold in the KSA. The date cereal lacked certain amino acids confirming the lower value of the protein digestibility-corrected amino acid score (PDCAAS) for DFPE (70%) compared to skimmed milk powder (100%) (**Chapter 2**). The minerals iron and zinc for date cereal also matched that of CERELAC but the calcium content was lower. The results indicate that the date cereal would have to be improved to be a competitor to the CERELAC product in the KSA. The calcium content could be improved by supplementation with calcium. The PDCAAS has to be increased to match that of CERELAC. This can only be achieved by further optimising the extraction of DFPE to obtain a more concentrated protein extract. The lack of amino acids could be supplemented by addition of camel milk.

A date cereal porridge prepared with camel milk had a proximate and mineral composition matching that of a porridge made with CERELAC prepared with cow's milk apart from lacking fat and calcium. The amino acid composition of the date cereal porridge waste similar to CERELAC porridge apart from leucine providing the RNI for infants under the age of 5 years old. Furthermore, instructions should be given on the packaging to make the porridge with camel milk. If, these instructions are followed, the 100 g portion of date cereal porridge would provide essential amino acids and minerals similar to CERELAC porridge, but with lower calorific value and lacking leucine. If the date cereal is supplemented with leucine, then it could be a competitor to CERELAC.

A concerning factor was that the date cereal porridge was perceived as significantly more bitter than the control porridge, although it was still within acceptable range (separate conversation by sensory panellists). Considering that the sensory panel consisted of adults, the bitterness might not be acceptable for infants which would reduce the potential for commercial uptake of the date cereal product as infant food. The bitterness might be due to the presence of oxalate and tannins in DFPE which are the compound responsible for the sharp taste in spinach and rhubarb. Further development to produce a purer form of DFPE with reduced oxalate and tannins would enhance its potential for application in baby food. The date cereal product made with camel milk would offer the advantage to CERELAC porridge that it is gluten-free and free of cow's milk, thereby it could be labelled as less allergenic than CERELAC porridge. It has also excellent anti-oxidant properties similar to vitamin C.

DFPE could be combined with other emerging plant protein concentrated, such as potato and pea protein to optimise the nutritional profile and PDCAAS value and could be applied to variety of commercially feasible product applications, such as nutrition bars targeted at pregnant women or the elderly. Results of functional

properties of DFPE reported in **Chapter 3** indicate that DFPE could be applied in beverages with neutral pH, soups, cake batters and vegetarian sausages. DFPE would also lend excellent anti-oxidant properties (**Chapter 2**) to the respective food products.

The date cereal product could be a competitor to CERELAC in the KSA as the country is the second largest producer of date palm fruit globally. People in this country consume date fruit daily and also accept to drink camel milk as they know very well the benefits of date fruit and camel milk.

Overall conclusion

KSA generates more than 200,000 tons of date palm biomass every year (Zafar, 2018) and more than 236,807 tons of dates are discarded each year in Algeria, resulting in environmental issues (Ahmed et al., 2016). If only these figures are considered, processing of the date fruit based on the extraction process in the present study, would lead to annual protein concentrate of 800 tons and 114 000 tons syrup in the KSA and 947 tons protein extract and 134, 979 tons syrup in Algeria.

The results of this study indicate that although DFPE could not fully replace SPI in respect to PDCAAS and functional properties, implementation of the extraction process would lead to reduction of waste of date fruit and contribute to the production of alternative protein sources to replace soy protein. The results of this study provide the basis of a process for protein extraction from date fruit, which could be further improved to maximise its usefulness to the food industry.

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